# THESE

présentée

A L'UNIVERSITE DE PARIS-SUD

CENTRE D'ORSAY

pour obtenir

LE GRADE DE DOCTEUR ES-SCIENCES NATURELLES

par

**Michel DAUÇA**

Sujet de la Thèse

**Prolifération et différenciation des cellules souches de l'épithélium intestinal des Amphibiens Anoures, lors des métamorphoses naturelle et induite.**

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soutenue le 28 Mai 1982, devant la commission d'examen

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LISTE DES PUBLICATIONS

(1) Remaniements de l'intestin des larves du Crapaud-accoucheur (Alytes obstetricans) lors d'une métamorphose spontanée ou induite par la thyroxine. Etude morphométrique et évolution du rapport protéines/ADN.
Dauça, M. et Hourdry, J., 1981 a. C. R. Acad. Sc. Paris, 292, 69-72.

(2) Cytological and cytochemical changes in the intestinal epithelium during anuran metamorphosis.
Hourdry, J. et Dauça, M., 1977. Internation. Rev. Cytol., supplt 5, 337-385.

(3) Isolement de l'épithélium intestinal chez le Crapaud-accoucheur à l'état larvaire ou juvénile.
Dauça, M. et Hourdry, J., 1978 a. Biol. Cell., 33, 85-88.

(4) Analyse quantitative des remaniements intestinaux et évolution de la synthèse de l'ADN. Etude chez les larves d'Alytes obstetricans et de Discoglossus pictus (Amphibiens Anoures) en métamorphose spontanée.
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(5) Effets in vivo et in vitro de la thyroxine sur l'épithélium intestinal des Amphibiens Anoures.
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(6) Analyse quantitative des remaniements intestinaux et évolution de la synthèse de l'ADN; Etude chez les larves d'Alytes obstetricans (Amphibien Anoure) traitées par la thyroxine.
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(9) Amphibian intestinal brush border enzymes during thyroxine-induced metamorphosis. A biochemical and cytochemical study.
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(14) Development of peroxisomes in Amphibians. II. Cytochemical and biochemical studies on the liver, kidney ans pancreas.
Dauça, M., Calvert, R., Ménard, D., Hugon, J.S. et Hourdry, J.
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REMANIEMENTS DE L'INTESTIN DES LARVES DU CRAPAUD-ACCOUCHEUR (*ALYTES OBSTETRICANS*) LORS D'UNE METAMORPHOSE SPONTANEE OU INDUITE PAR LA THYROXINE. ETUDE MORPHOMETRIQUE ET EVOLUTION DU RAPPORT PROTEINES/ADN.

Comptes-rendus de l'Académie des Sciences de Paris, *292*, 69-72 (1981).

ZOOLOGIE DES VERTÉBRÉS. – *Remaniements de l'intestin des larves du Crapaud-accoucheur* (Alytes obstetricans) *lors d'une métamorphose spontanée ou induite par la thyroxine. Étude morphométrique et évolution du rapport protéines/ADN.* Note (*) de **Michel Dauça** et **Jacques Hourdry,** présentée par Pierre-Paul Grassé.

L'intestin des larves du Crapaud-accoucheur (*Alytes obstetricans*) subit d'importants remaniements, au cours de la métamorphose spontanée. A partir du stade XVIII, les rapports : poids de l'intestin/poids total et longueur de l'intestin/longueur corporelle diminuent. Les diminutions maximales présentées par ces paramètres sont respectivement de 83 et de 90 % au stade XXIII. La régression de la queue ne commence qu'à partir du stade XX. Pour l'intestin, le rapport protéines/ADN culmine au stade XIX, chute brutalement au stade XX et réaugmente faiblement au stade XXII.

Chez les larves traitées par la thyroxine, les transformations intestinales ne débutent qu'après une latence de 36-48 h. La régression caudale est plus tardive. Le rapport protéines/ADN, déterminé pour l'intestin des têtards traités, reste sensiblement constant et voisin de celui observé chez les larves témoins.

*During spontaneous metamorphosis of* Alytes obstetricans *tadpoles (Anuran Amphibia), the gut undergoes important modifications. The gut weight/total weight and gut length/body length decrease by stage XVIII. They reach maximum reductions of* 83 *and* 90 %, *respectively, by stage XXIII. Tail regression begins only by stage XX. The intestinal protéin/DNA ratio peaks to a maximum by stage XIX, decreases greatly by stage XX then increases slightly by stage XXII.*

*During thyroxine-induced metamorphosis, the morphometric changes are observable after a lag period of* 36-48 *hs, prior to detectable tail reduction. The intestinal protein/DNA ratio of* $T_4$*-treated tadpoles remains constant and no difference with control data is noted.*

INTRODUCTION. – Au climax de la métamorphose, ou paroxysme du phénomène, l'épithélium intestinal des Amphibiens Anoures subit des phénomènes d'histolyse et d'histogénèse : l'épithélium larvaire (épithélium primaire) dégénère, tandis qu'une nouvelle assise (épithélium secondaire) se met en place [1]. Ces processus sont contemporains d'importants changements morphologiques. L'intestin est long et spiralé chez le têtard, mais il devient court et subrectiligne chez les juvéniles. Si la séquence des événements histologiques a été très étudiée au niveau de l'intestin ([2], [3]), peu de travaux concernent les variations morphométriques présentées par l'organe ([4], [5]). Dans le présent travail, nous avons quantifié ces variations et celles du rapport protéines/ADN, dans l'intestin du têtard du Crapaud-accoucheur au cours d'une métamorphose naturelle ou induite par la thyroxine.

MATÉRIELS ET MÉTHODES. – *Animaux.* – Les larves du Crapaud-accoucheur (*Alytes obstetricans*), récoltées dans diverses lavognes du Larzac, sont élevées à la température de 20°C et nourries *ad libitum*. L'étude de l'intestin chez les animaux en métamorphose naturelle a été réalisée au printemps, le stade de développement étant apprécié au moyen de la table de Taylor et Kollros [6]. Le traitement par la thyroxine est effectué en hiver.

*Traitement par la thyroxine* ($T_4$). – Les larves sont choisies en fin de prémétamorphose (stades X-XII). Les témoins sont conservés dans l'eau de source. Les animaux traités sont placés dans de l'eau de source additionnée de thyroxine (Roche) à la concentration de $10^{-7}$ g/ml. Cette solution est renouvelée tous les 2 jours.

*Morphométrie.* – Les animaux sont séchés puis pesés. Leur longueur totale et celle de leur queue sont mesurées. Les larves sont ensuite sacrifiées. L'intestin est prélevé de l'arrivée du canal cholédoque au rectum, déroulé dans du tampon Tris (2mM) mannitol (50 mM) à pH 7,4 et à 4°C. La longueur de l'organe est déterminée après étalement sur une plaque de verre refroidie. L'intestin est ouvert longitudinalement, vidé de son contenu dans le tampon, séché et pesé. Six à huit animaux sont utilisés pour chaque détermination morphométrique.

*Biochimie.* – L'intestin est broyé, à 4°C, dans le tampon Tris-mannitol. Le dosage des protéines de l'homogénat est réalisé selon la méthode de Lowry et coll. [7]. L'ADN est quantifié grâce à la technique de Burton [8] modifiée par Giles et Myers [9].

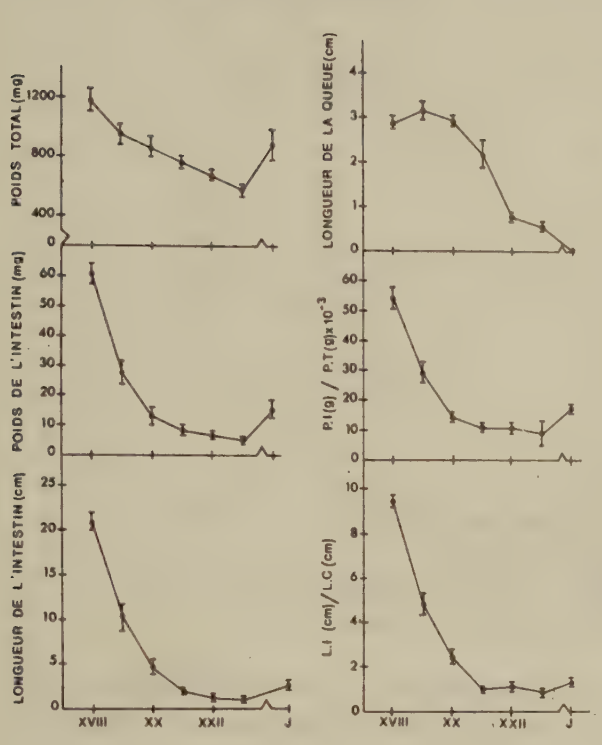


Fig. 1

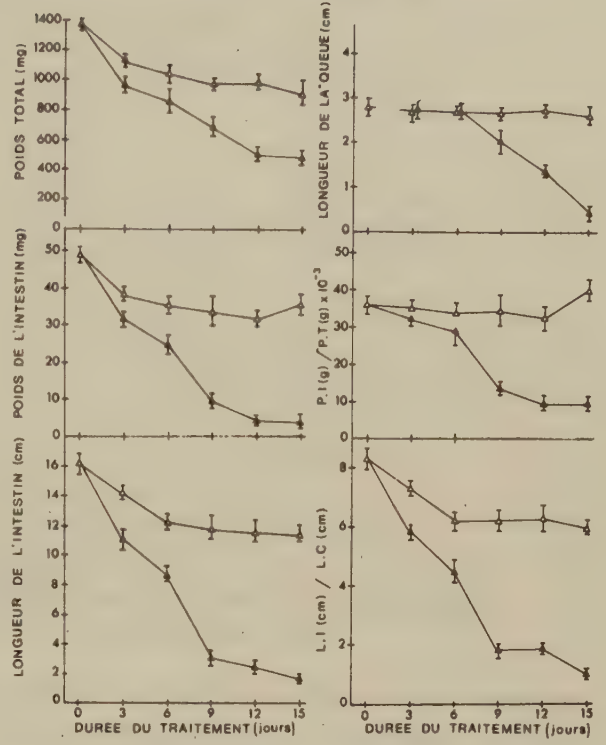


Fig. 2

Fig. 1. – Étude morphométrique des remaniements intestinaux au cours de la métamorphose naturelle (J : stade juvénile).

Fig. 2. – Traitement des larves au stade X-XII par $T_4$ ($10^{-7}$ g/ml). Analyse morphométrique des transformations intestinales. (△), témoin; (▲), larve traitée.

RÉSULTATS. – *Morphométrie.* – *Métamorphose spontanée :* Dans la mesure où la taille maximale des larves est atteinte au stade XVIII nos observations ont porté sur les seules modifications postérieures à ce stade et se produisant au climax de la métamorphose. Le poids total de l'animal (P.T.), le poids de l'intestin (P.I.) et sa longueur (L.I.), la longueur de la queue (L.Q.), les rapports P.I./P.T. et L.I./L.C. (L.C. = longueur du corps) subissent alors une diminution évidente (*fig.* 1). Contrairement aux autres paramètres qui s'abaissent dès le stade XVIII, la réduction caudale ne devient apparente qu'après le stade XX.

*Métamorphose induite par* $T_4$. – La thyroxine exogène entraîne une réduction des paramètres : P.T., P.I, L.I., P.I./P.T. et L.I./L.C., que l'on peut apprécier dès le 3$^e$ jour (*fig.* 2). Par contre, la diminution de la longueur caudale ne devient effective qu'après 6 à 9 jours de traitement hormonal. Durant les 2 premiers jours du traitement, les mesures morphométriques ont été effectuées toutes les 12 h. L'examen de la figure 3 montre que le poids et la longueur de l'intestin des larves traitées demeurent voisins des valeurs témoins durant les premières 48 h, ce qui accrédite l'existence d'une latence d'action de $T_4$ au niveau intestinal.

*Biochimie.* – *Métamorphose spontanée :* Le rapport : protéines/ADN augmente fortement durant les stades XVIII-XIX, chute au stade XX, s'accroît faiblement au stade XXII avant de retrouver chez les individus juvéniles une valeur comparable à celle qui est calculée chez les larves au stade XII (*fig.* 4 A).

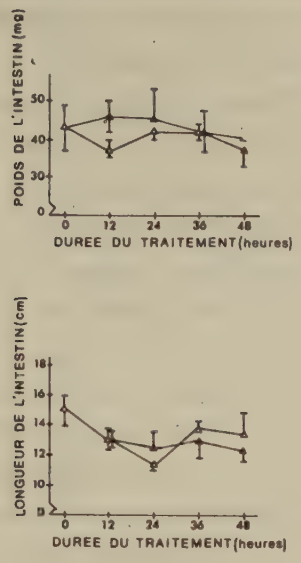


Fig. 3

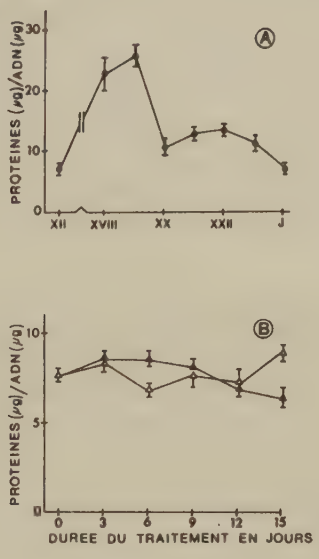


Fig. 4

Fig. 3. – Étude de la latence dans la réponse de l'intestin à l'action de $T_4$. (△), témoin; (▲), larve traitée.

Fig. 4. – Évolutions du rapport protéines/ADN; (A) lors d'une métamorphose spontanée, (B), ou induite par la thyroxine. (△), témoin; (▲), larve traitée.

*Métamorphose induite.* – Le rapport : protéines/ADN, déterminé à divers stades du traitement hormonal par $T_4$ ne diffère pas des valeurs obtenues chez les témoins (*fig.* 4 B).

Discussion. – Le développement postembryonnaire des Amphibiens Anoures est classiquement divisé en deux étapes : prémétamorphose et métamorphose. La prémétamorphose est une période longue, caractérisée par une croissance progressive de la plupart des organes de la larve. Pendant la métamorphose et spécialement durant le climax surviennent d'importants changements morphologiques, histologiques et biochimiques qui transforment rapidement une larve dulçaquicole, herbivore, en un individu juvénile, terrestre et carnivore. Les remaniements subis par l'intestin des têtards sont particulièrement spectaculaires. Rapporté à la longueur initiale de l'intestin larvaire, le raccourcissement constaté correspond à une diminution de l'ordre de 68 % chez *Rana pipiens* [10], de 84 % chez *Rana catesbeiana* [4] et de 94 % chez *Alytes obstetricans*. Cette involution s'amorce au stade XVIII et atteint son paroxysme au stade XXII. Une légère augmentation du poids et de la longueur de l'intestin est toutefois observée chez les juvéniles. La valeur maximale atteinte par le rapport protéines/ADN au stade XIX reflète la manifestation des processus histolytiques dans l'épithélium primaire, avec disparition des noyaux et une diminution de la teneur de l'ADN dans le tissu, alors que les protéines plus ou moins hydrolysées ne sont que partiellement éliminées. La chute de ce rapport au stade XX est liée à l'élévation du taux d'ADN dans l'épithélium, consécutive à la prolifération intense des cellules souches de l'épithélium secondaire ([11], [12], [2], [3]). Au stade XXII, les divisions cellulaires sont moins fréquentes et les épithéliocytes secondaires en se différenciant synthétisent activement des protéines de structure et des enzymes dont celles de la nouvelle bordure en brosse intestinale ([13] à [17]). Ces changements peuvent expliquer la légère augmentation du rapport protéines/ADN au stade XXII.

Le traitement des larves d'Anoures par $T_4$ ne provoque pas de modifications évidentes du rapport protéines/ADN vis-à-vis des témoins, contrairement à ce que l'on observe en métamorphose naturelle. Un tel résultat peut s'expliquer par le fait que, dans ce dernier cas,

la concentration plasmatique des hormones thyroïdiennes croît progressivement et déclenche les phénomènes d'histolyse intestinale avant les phénomènes d'histogénèse. D'autre part, il est fort probable que l'action de $T_3$ et$T_4$ soit renforcée par celle d'autres hormones, dont celles des cellules interrénales. Au contraire, lors du traitement par la thyroxine, les têtards sont d'emblée soumis à une dose importante et non physiologique d'hormone. Dès lors, histolyse et histogénèse se produisent simultanément et assurent la constance du rapport protéines/ADN.

Lors de la métamorphose, les concentrations plasmatiques des hormones thyroïdiennes ont été estimées au moyen de techniques radio-immunologiques ([18] à [24]). Tous ces travaux confirment l'existence de concentrations plasmatiques maximales des hormones au climax de la métamorphose. En première approximation, $T_4$ semble aussi efficace que $T_3$ dans le déclenchement de la métamorphose intestinale. En effet, l'une et l'autre hormones sont capables d'assurer la transformation de l'intestin larvaire en un organe adulte; de plus, la latence observée avec la $T_4$ est proche de celle obtenue avec la $T_3$ [4]. La recherche de la fréquence des récepteurs cytoplasmiques et nucléaires spécifiques de la $T_3$ et de la $T_4$, dans les épithéliocytes intestinaux, pourrait néanmoins préciser l'efficacité respective de chacune de ces deux hormones, lors des remaniements de l'épithélium intestinal.

Ce travail sur la « Métamorphose des Amphibiens » a été réalisé dans le cadre de l'Équipe de Recherches associées au C.N.R.S. (E.R.A. n° 070902). Excellente collaboration technique de M^mes C. Niederst et E. Rodier.

(*) Remise le 17 novembre 1980.

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*Laboratoire Biologie et Biochimie du Développement,*
*École normale supérieure, grille d'Honneur,*
*parc de Saint-Cloud,* 92210 *Saint-Cloud*
et *Laboratoire Biologie-Vertébrés,*
*Centre d'Orsay, Université de Paris-Sud,* 91405 *Orsay.*

CYTOLOGICAL AND CYTOCHEMICAL CHANGES IN THE INTESTINAL EPITHELIUM DURING ANURAN METAMORPHOSIS.

International Review of Cytology, Supplément n°5, 337-385 (1977).

# Cytological and Cytochemical Changes in the Intestinal Epithelium during Anuran Metamorphosis

J. HOURDRY

*Laboratoire de Biologie-Vertébrés, Centre d'Orsay, Université Paris-Sud, Orsay, France; Laboratoire de Microscopie Électronique Appliquée à la Biologie, CNRS, Paris, France*

AND

M. DAUCA

*Laboratoire des Sciences Naturelles de l'Ecole Normale Supérieure de St. Cloud, St. Cloud, France*

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## I. Introduction

The passage of anuran amphibians from the aquatic to the terrestrial life is accompanied by a change in the organizational plan of the animal, which defines metamorphosis. Various modifications visible to the naked eye reflect histolytic and histogenetic events, whereas others occur at the molecular level, as demonstrated by retinal or respiratory pigment substitutions (Frieden and Just, 1970).

The gut undergoes important alterations during metamorphosis. The initial or primary epithelium degenerates and is replaced by a layer of

neoformation or secondary epithelium. These transformations were reported in some of the earliest publications (Bataillon, 1891; Morse, 1918; Kuntz, 1924; Janes, 1934; Joly, 1937) and more recently by Bonneville (1963), Bernard (1965), Hassan and Marinucci (1966), Brown and Millington (1968), Kopéc (1970), and Hourdry (1972a).

The first part of this article considers the intestinal epithelium of larvae undergoing natural development. Under such conditions, lysosomes have been observed before and during the degeneration of primary epithelium cells. These organelles are generally associated with alterative phenomena (de Duve and Wattiaux, 1966; Ericsson, 1969a) and contain various lytic hydrolases.

Anuran metamorphosis is due to an increase in thyroid activity, the mechanisms of which have been investigated in detail by Etkin (1968). The second portion of this article examines the effects of thyroid hormones (triiodothyronine, $T_3$, and thyroxine, $T_4$) on intestinal epithelial cells analyzed following experimental alteration of the level of circulating hormones.

## II. The Intestinal Epithelium of the Larva during Natural Development

Three periods have been distinguished by Etkin (1968), the first, premetamorphosis, being a simple growth phase. The second, prometamorphosis, signals the climax, during which the majority of metamorphic events accelerate.

The most often utilized larvae are those of *Rana catesbeiana, Rana temporaria, Alytes obstetricans, Discoglossus pictus,* and *Xenopus laevis*. The degree of development of the larvae of the first four species is estimated most often with the table presented by Taylor and Kollros (1946). Thus premetamorphosis corresponds to stages I through XI, during which the hindlimbs remain rudimentary. Stages XII through XIX define prometamorphosis, during which the hindlimbs develop. Then the cloacal tube disappears (Stage XVIII), and the forelimbs become prominent under the skin (Stage XIX). Climax occurs between stages XX and XXV; one forelimb appears (Stage XX), and then the other (Stage XXI); the tail regresses and disappears completely by stage XXV.

The degree of development of *Xenopus* larvae is outlined in the table published by Nieuwkoop and Faber (1956), in which prometamorphosis corresponds to stages 56 through 59, and climax to stages 60 through 66.

During pre- and prometamorphosis, the intestine is long and spiral and has a large diameter (Fig. 1A). At the onset of climax, it becomes

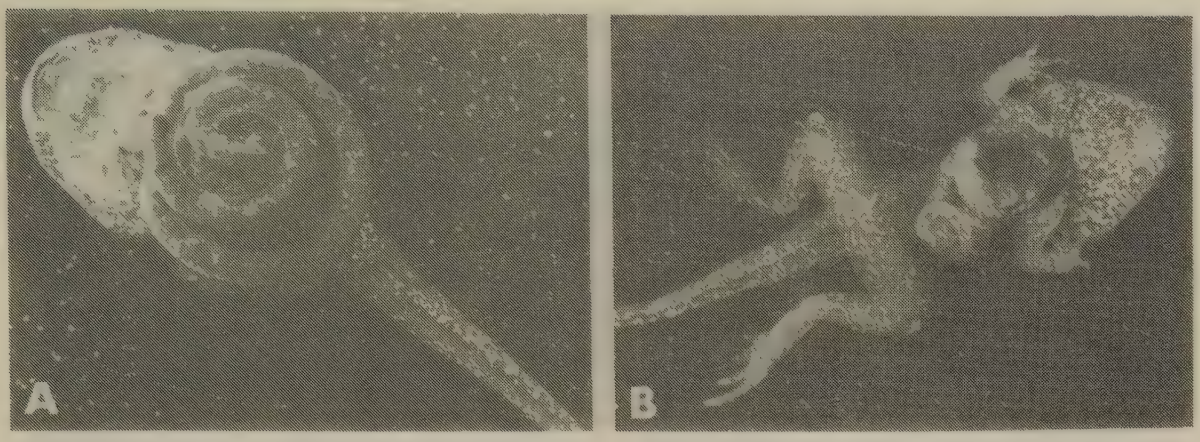



FIG. 1. Intestine of a *Discoglossus* larva. (A) Stage XII. ×2. (B) Stage XXI. ×2.

short, and its cross section decreases (Fig. 1B). Important epithelial alterations are associated with these morphological modifications, beginning at the end of prometamorphosis.

### A. Intestinal Primary Epithelium before Changes

Intestinal primary epithelium has been observed in tadpoles in premetamorphosis (Fig. 2) and in the initial stages of prometamorphosis (Figs. 3 and 4; Bonneville, 1963; Hourdry, 1969a). The mitochondria are localized in the supranuclear space of the epithelial cells, and the Golgi apparatus is only slightly differentiated. At the beginning of prometamorphosis, lysosomes less than 1 $\mu$m wide are observed in the wider supranuclear zone (Fig. 4). Some of these lysosomes are autolytic vacuoles in which sequestered organelles can be recognized, mostly cisternae of the endoplasmic reticulum. The other lysosomes are electron-opaque (dense bodies) or contain membrane whorls (myeloid bodies). They may represent a population of residual bodies resulting from the evolution of autolytic vacuoles. Also, at the beginning of prometamorphosis, the rough endoplasmic reticulum becomes organized, occasionally in parallel cisternae.

Several stem cells are intercalated at the base of the epithelium (Fig. 3), which has a thin, cytoplasmic fringe.

### B. Histolytic Phenomena

Intense degeneration occurs in intestinal primary epithelium at the end of prometamorphosis and especially at the onset of climax (Bonneville, 1963; Hourdry, 1969b, 1971a).

#### 1. *Lysosomes*

a. *Principal Types*. In midprometamorphosis, lysosomes become more numerous in the primary epithelium cells, although their diame-

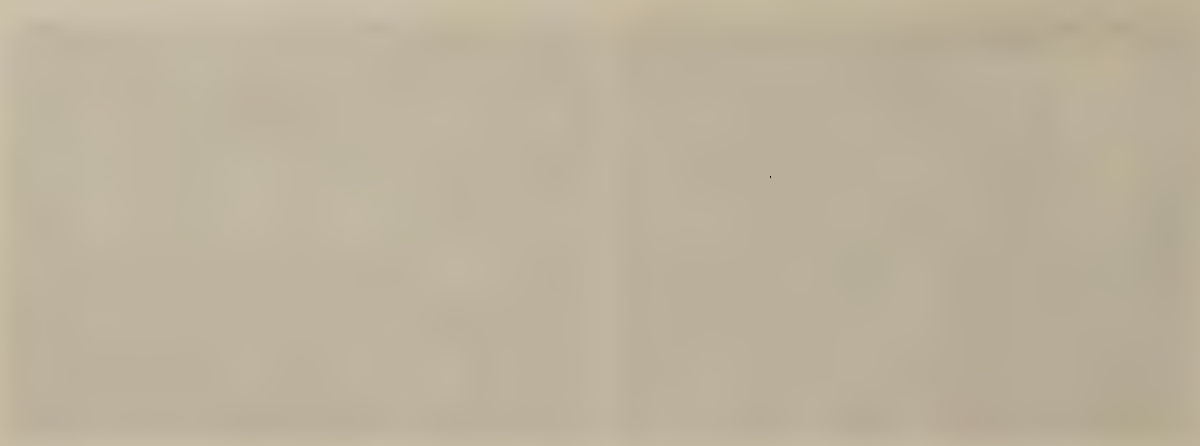


FIG. 2. *Discoglossus*, stage V. Rare multivesicular bodies (mvb) are localized in the supranuclear zone. At bottom right an outcropping of connective tissue (ct) can be seen. bb, Brush border; G, Golgi apparatus. ×8000. (From Hourdry, 1971d.)

ter still rarely exceeds 1 $\mu$m. Several have dense, granular, and homogeneous contents and have undoubtedly never participated in lytic phenomena ["virgin lysosomes" as described by Moe *et al.* (1965)]. Others are dense or myeloid residual bodies.

From the end of prometamorphosis onward, more voluminous lysosomes appear in the primary epithelium cells. They are recognized with the light microscope by their high degree of chromophilicity (Fig. 5A and B). When examined with the electron microscope, these

lysosomes exhibit characteristics of autolytic vacuoles and residual bodies. They have been classified into four categories in several anuran species (Hourdry, 1971a):

1. *Primary autolytic vacuoles* (Fig. 6A–D). No larger than 4 $\mu$m in diameter, they are bounded by one or two membranes. They contain dilated mitochondria, reticular cisternae, or ribosomes. Some vacuoles also contain Golgi-like vesicles.
2. *Primary residual bodies* (Fig. 6E). During degeneration, the organelles segregated within the primary autolytic vacuoles are identified with more difficulty. The intravacuolar material generally becomes opaque, and membrane whorls develop. The vacuoles are transformed into dense or myeloid primary residual bodies, reach a diameter of 4 $\mu$m, and most often are limited by a single membrane.
3. *Secondary autolytic vacuoles* (Fig. 7). Their diameter is greater than 6 $\mu$m. In addition to various organelles, primary autolytic vacuoles and their residual forms are also segregated within the secondary autolytic vacuole.

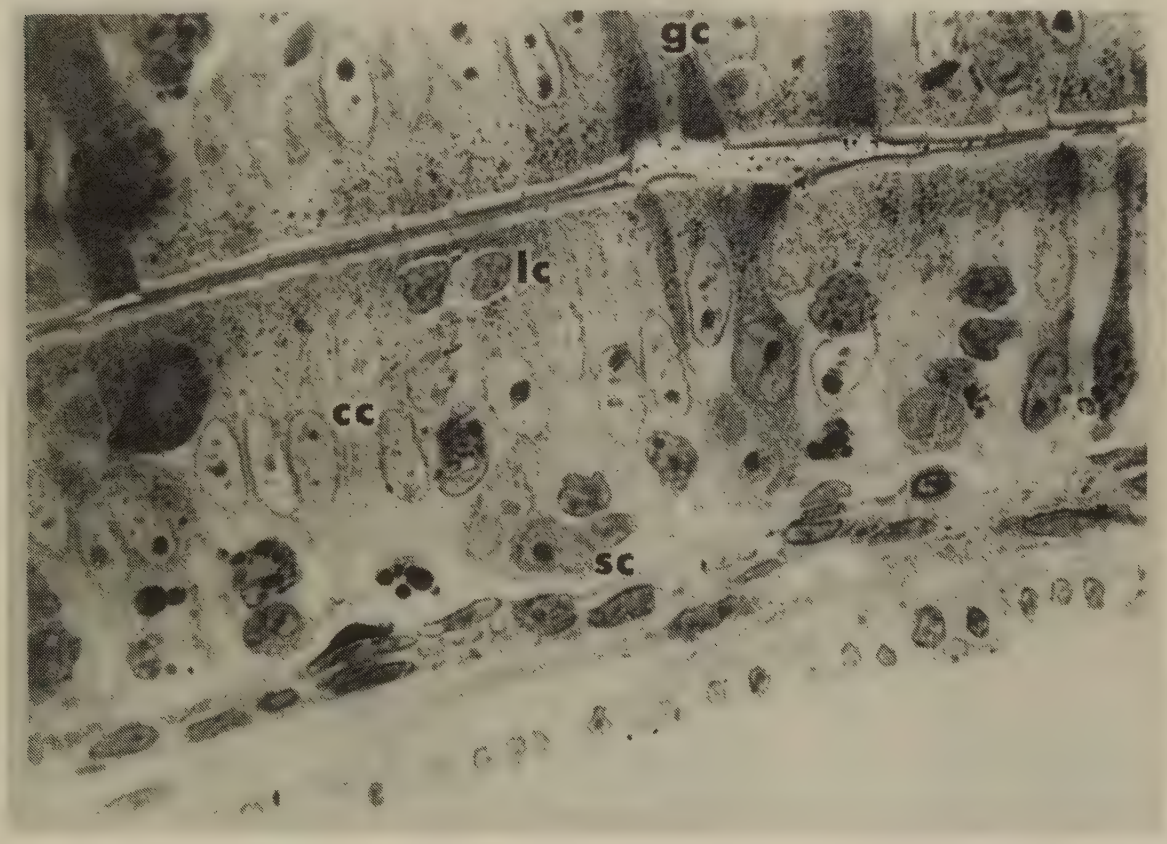
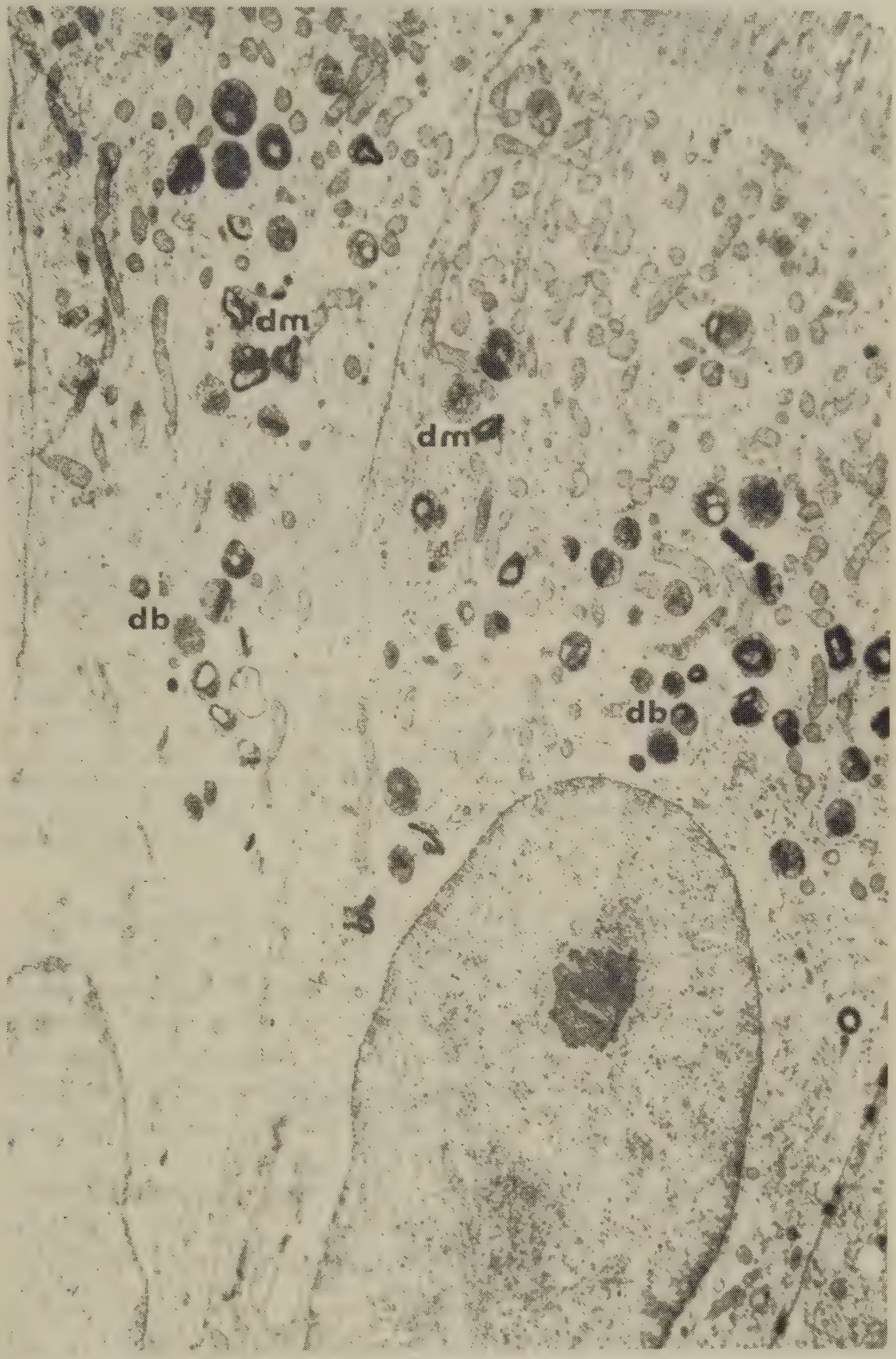



FIG. 3. *Alytes*, stage XIII. Semithin section of the gut wall. Several goblet cells (gc) and lymphocytes (lc) are seen between the columnar cells (cc) of the primary epithelium. A stem cell (sc) is occasionally seen at the base of the tissue. ×1150. (From Hourdry, 1969a.)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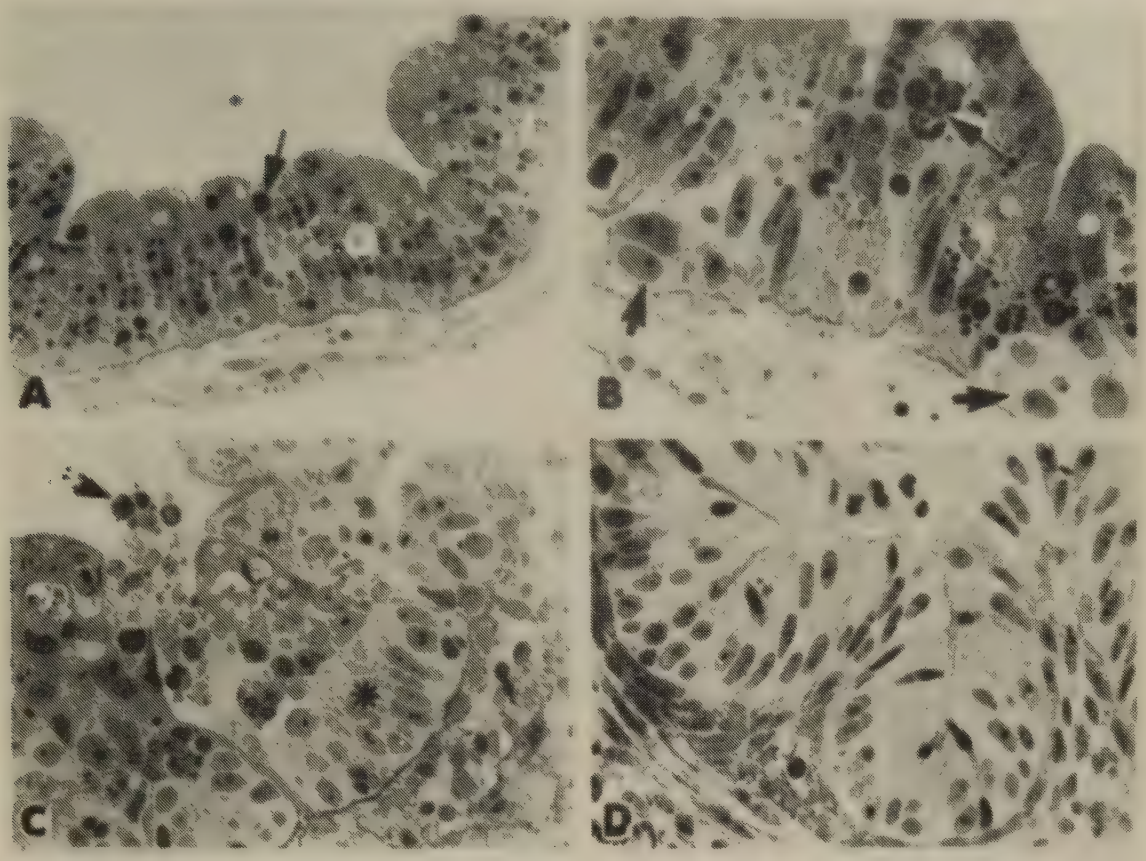


FIG. 5. (A and B) *Discoglossus,* stage XIX. The primary epithelium contains large chromophilic lysosomes (thin arrows). Two small islets of embryonic cells are located under the primary epithelium cells (thick arrows). (A) ×150. (B) ×350. (C) *Discoglossus,* stage XX-XXI. Basal stem cells have multiplied (asterisk) under the degenerating primary epithelium, the remnants of which are passing into the lumen (arrow). ×350. (D). *Discoglossus,* stage XXII. The secondary epithelium is composed of villi. ×350. (From Hourdry, 1971b.)

4. *Secondary residual bodies* (Fig. 8). The degenerative processes occurring within the secondary autolytic vacuoles no longer permit the primary residual bodies to be distinguished from the material surrounding them. The vacuoles become secondary residual bodies, enclosing membrane whorls or vesicular aggregates. Electron-transparent areas are sometimes observed within them, and the limiting membrane can partially disappear.

The relationship among the various lysosomes is summarized in Fig. 9.

Autolytic vacuoles and their residual forms have been observed in

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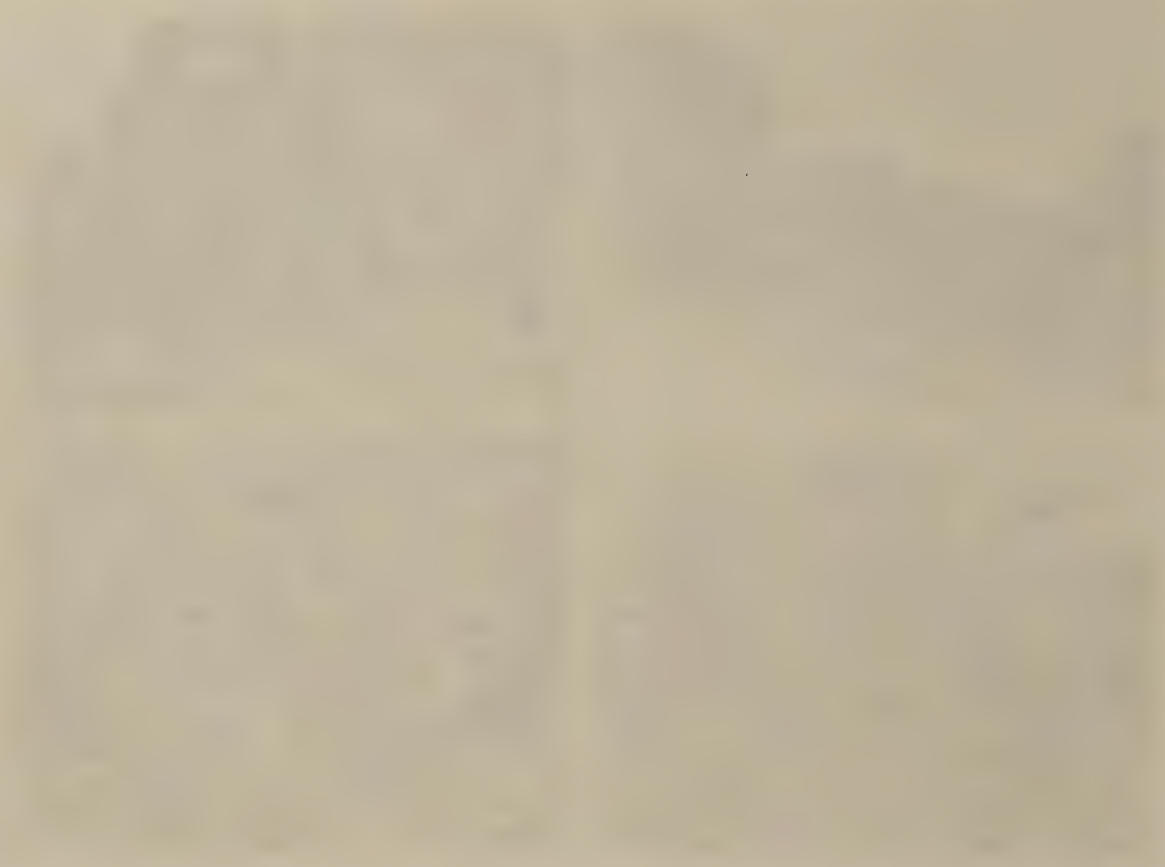
FIG. 4. *Alytes,* stage XIII. Columnar cells of the primary epithelium. Dense bodies (db) and myeloid bodies (mb) of small size are present in the enlarged supranuclear zone. ×9500. (From Hourdry, 1969a.)



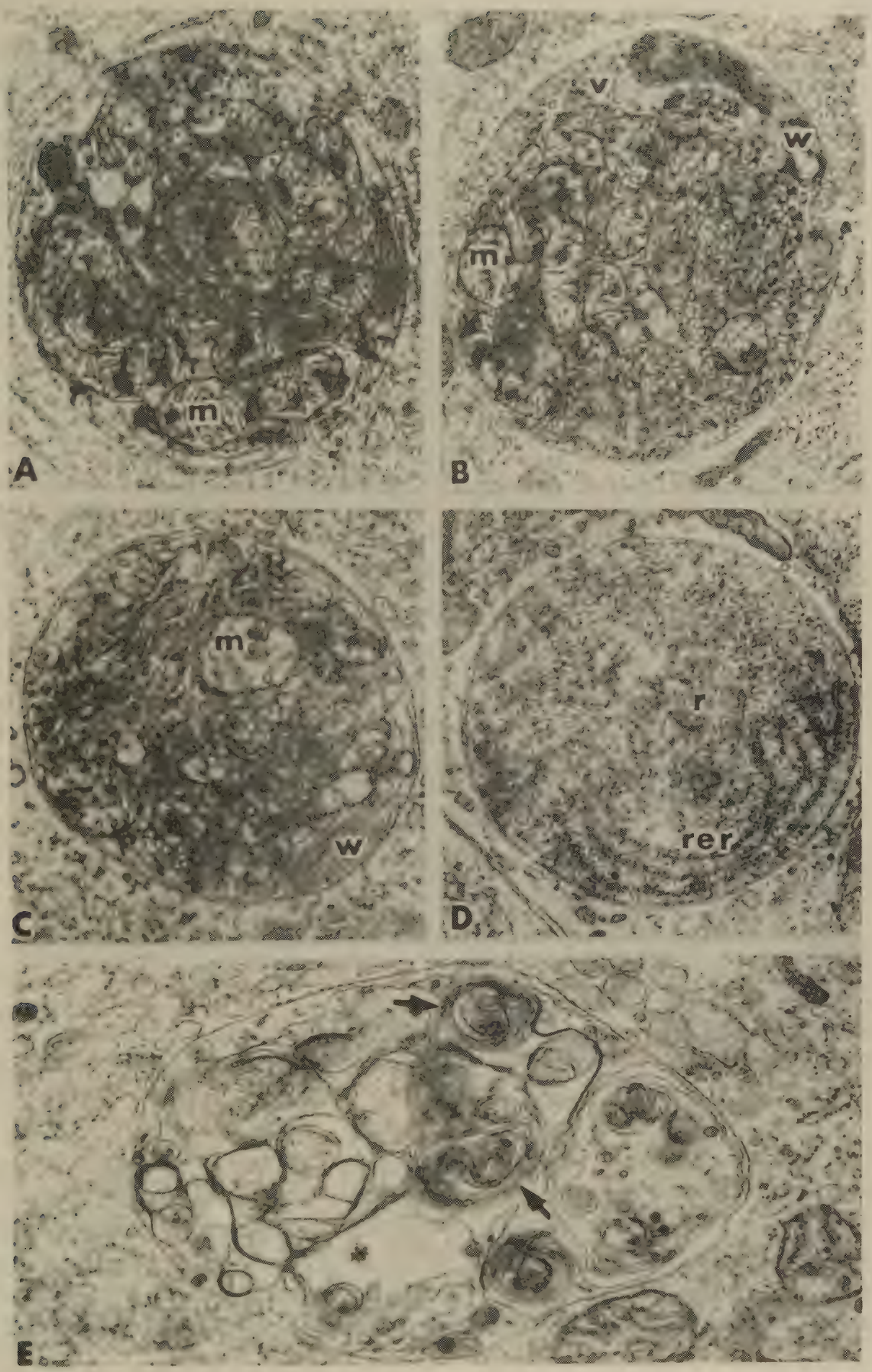



FIG. 6. (A–D) Primary autolytic vacuoles in which mitochondria (m), Golgi-like vesicles (v), rough endoplasmic reticulum cisternae (rer), and ribosomes (r) can be recognized. Membrane whorls are sometimes present (w). (A) *Xenopus*, stage 57.

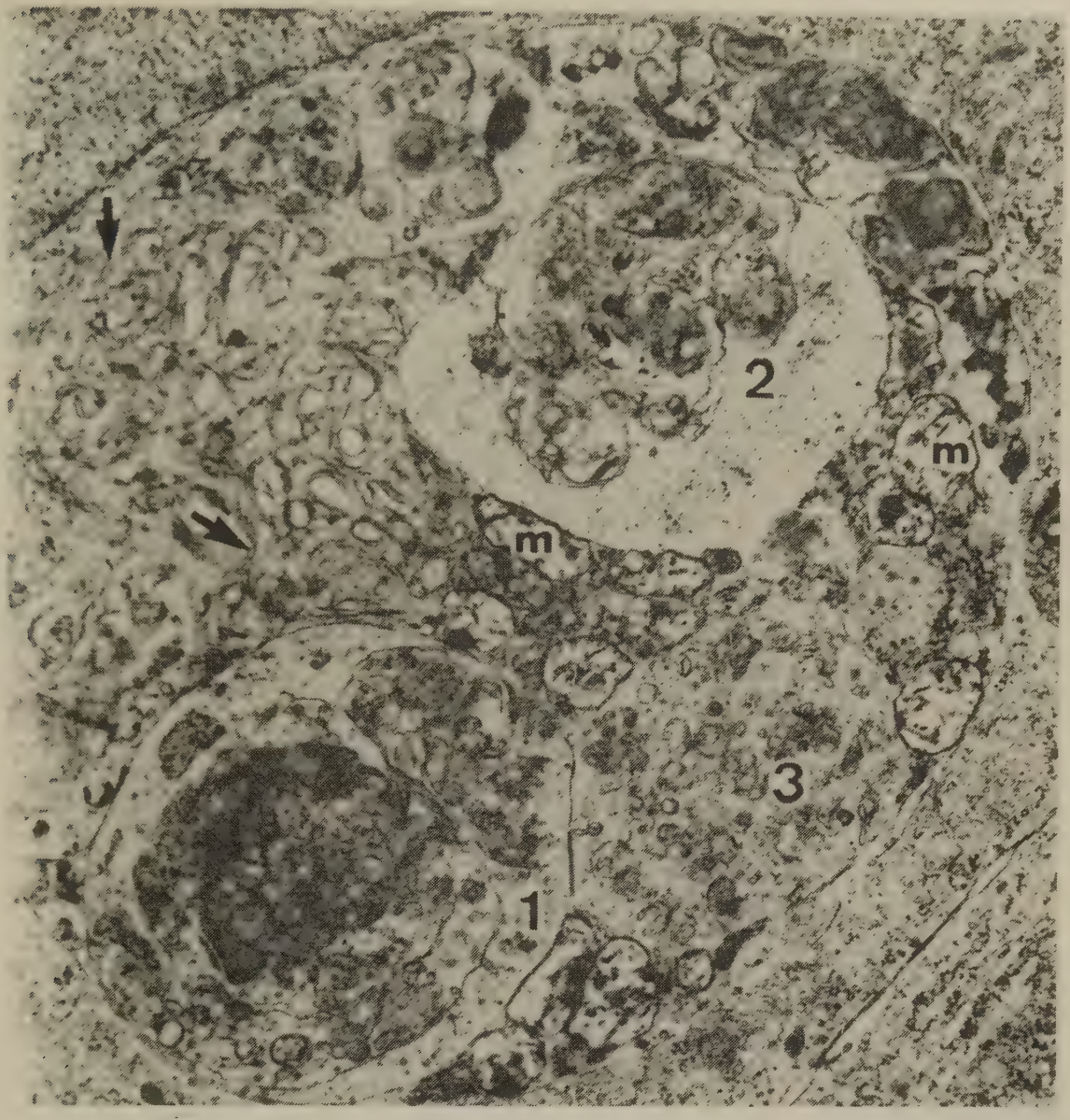



FIG. 7. *Discoglossus*, stage XIX. Secondary autolytic vacuole. Primary residual bodies 1 and 2 are still well individualized, but body 3 is easily confused with the rest of the vacuole. Several mitochondria (m) are still recognizable in the intermediate spaces, where large membrane whorls are noted (arrows). ×10,500. (From Hourdry, 1971a.)

many cells subjected to pathogenic conditions. They have also been seen in organs undergoing natural regression. Thus, in insect larvae, the presence of autolytic vacuoles is associated with involution of the prothoracic gland (Osinschak, 1966; Scharrer, 1966; Beaulaton, 1967), the salivary glands (Schin and Clever, 1968), the silk gland (Matsuura *et al.*, 1968), or the intestine (Misch, 1965). During anuran metamor-

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×12,000. (B–D) *Discoglossus*, stage XIX. (B) ×12,500. (C) ×22,500. (D) ×20,500. (E) *Discoglossus*, stage XX. Primary residual body containing membrane whorls (arrows). ×17,000. (From Hourdry, 1971a, 1972b.)

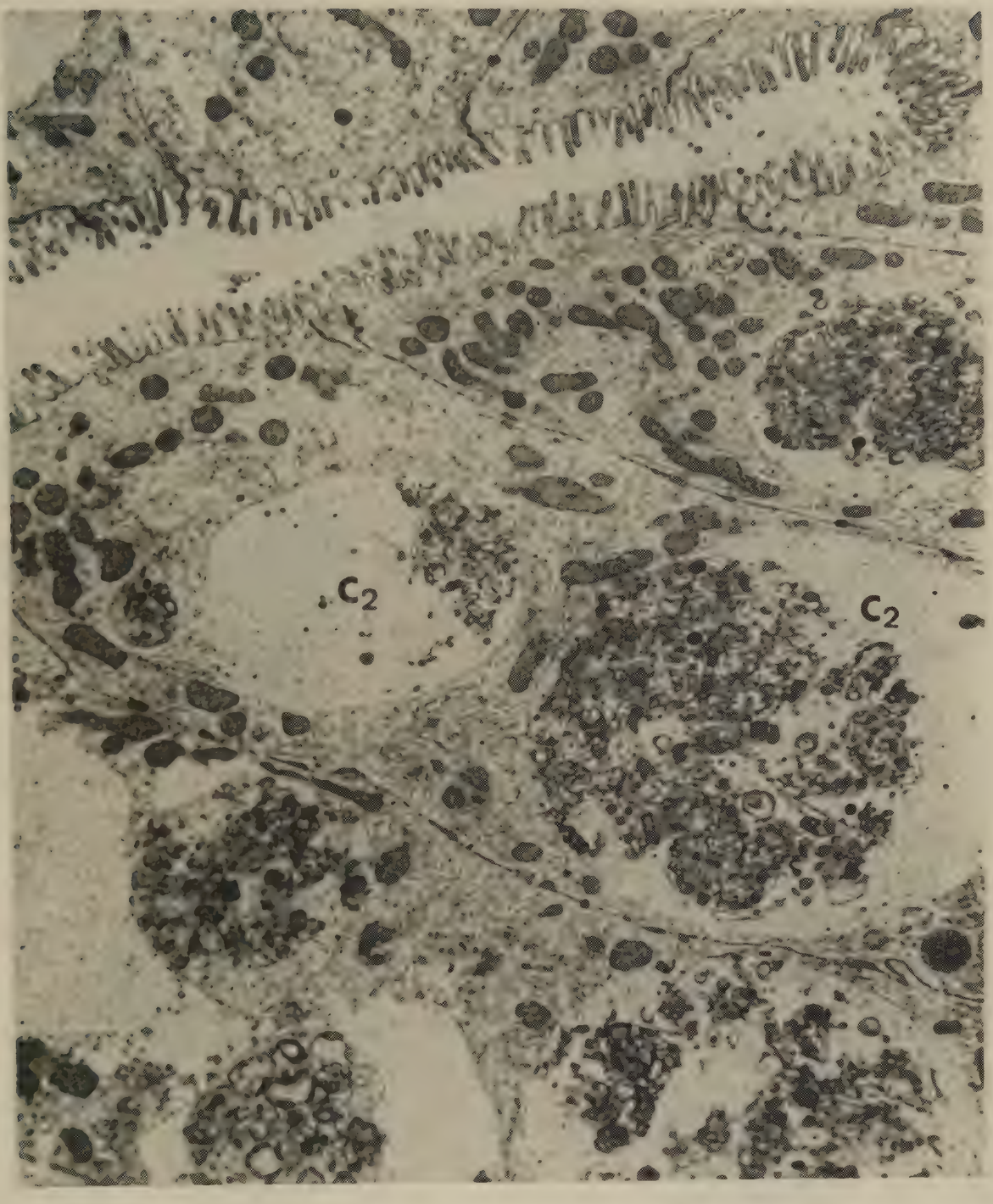



FIG. 8. *Discoglossus*, stage XX. Secondary residual bodies ($C_2$). ×4500. (From Hourdry, 1971a.)

phosis, these vacuoles have been described in several tissues undergoing alteration, including gill epithelium (Hourdry, 1974a) and various caudal tissues (Fox, 1973a,b).

b. *The Membrane of Autolytic Vacuoles and of Residual Bodies.* The origin of this membrane varies according to cell type. It can be of

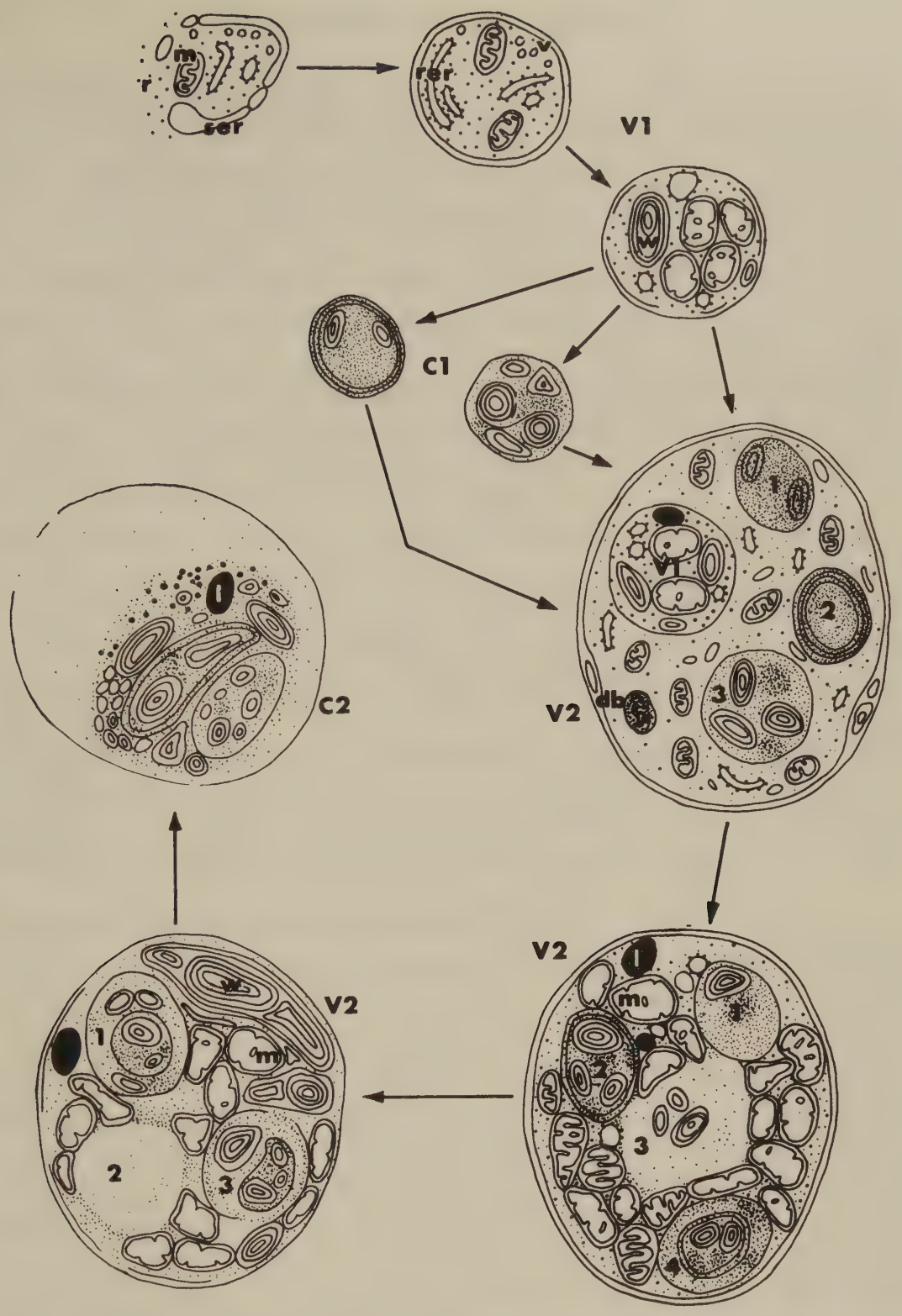



FIG. 9. Evolution of autolytic vacuoles in primary intestinal epithelium cells. The isolation of cytoplasmic constituents by cisternae of the smooth endoplasmic reticulum (ser) results in the appearance of primary autolytic vacuoles (V1). These vacuoles, as well as the primary residual bodies derived from them (C1), are segregated in the interior of large secondary autolytic vacuoles (V2). The latter are in turn transformed into secondary residual bodies (C2). db, Small dense body; l, lipid droplet; m, mitochondrion; r, ribosome; rer, rough endoplasmic reticulum; v, Golgi-like vesicle; w, membrane whorl; 1–4, primary residual bodies sequestered within secondary vacuoles. (From Hourdry, 1973a.)

Golgi origin, following the enclosure of a cytoplasmic region by a saccule (Frank and Christensen, 1968; Schiaffino and Hanzlíková, 1972a). A reticular origin has also been suggested. It has been reported that a portion of cytoplasm is surrounded by smooth endoplasmic reticulum (Novikoff and Shin, 1964; Swift and Hruban, 1964; Hugon and Borgers, 1965; Ericsson, 1969a; Schiaffino and Hanzlíková, 1972a) or rough endoplasmic reticulum (Beaulaton, 1967; Flickinger, 1968; Cole *et al.*, 1971). The reticular nature of the membrane has been demonstrated by the visualization of reticulum-specific enzymic activity (Arstila and Trump, 1969; Ericsson, 1969b; Vorbrodt *et al.*, 1971).

In addition, the membrane can appear *de novo* (Ashford and Porter, 1962; Napolitano, 1963; Policard, 1965).

Newly formed autolytic vacuoles in intestinal primary epithelium cells of anuran larvae often have a peripheral double membrane (Hourdry, 1972a). This excludes a *de novo* origin, which is compatible only with the immediate formation of a single membrane. Although segregation by enclosure in a Golgi saccule is rarely noted, several images suggest the closure of a profile of smooth endoplasmic reticulum. Degeneration of the contents of the vacuole is accompanied by the disappearance of the most internal parietal membrane. Separation of the two membranes has occasionally been observed, the inner membrane retracting around the material being engulfed.

2. *Highly Altered Primary Epithelial Cells*

Degeneration is no longer confined to the interior of the lysosome but involves all the cytoplasm in these cells. The brush border becomes irregular and disappears (Fig. 10), the mitochondria are often dilated and clear (Fig. 10), and the reticulum is formed of swollen cisternae and aggregates of small vesicles (Figs. 10 and 11). The Golgi vacuoles are generally electron-opaque (Fig. 10), and they contain osmiophilic granules between 300 and 1200 Å in diameter (Hourdry, 1969b). Lipid droplets are often seen in cells undergoing alteration (Fig. 11); they may represent some of the degradation products a lipoprotein membrane material gives rise to (Siekewitz, 1963). They could also represent a pool of lipids not utilized by cells whose protein synthetic capacity is altered and which are incapable of producing new lipoprotein material (Stein and Stein, 1967).

3. *The Fate of Material from Destroyed Cells*

a. *Liberation into the Intestinal Lumen.* The remnants of primary epithelium cells that have been destroyed are extruded into the intestinal lumen after the apical cell membrane is opened (Bonneville, 1963; Hourdry, 1969b). Although such observations are rare at the end

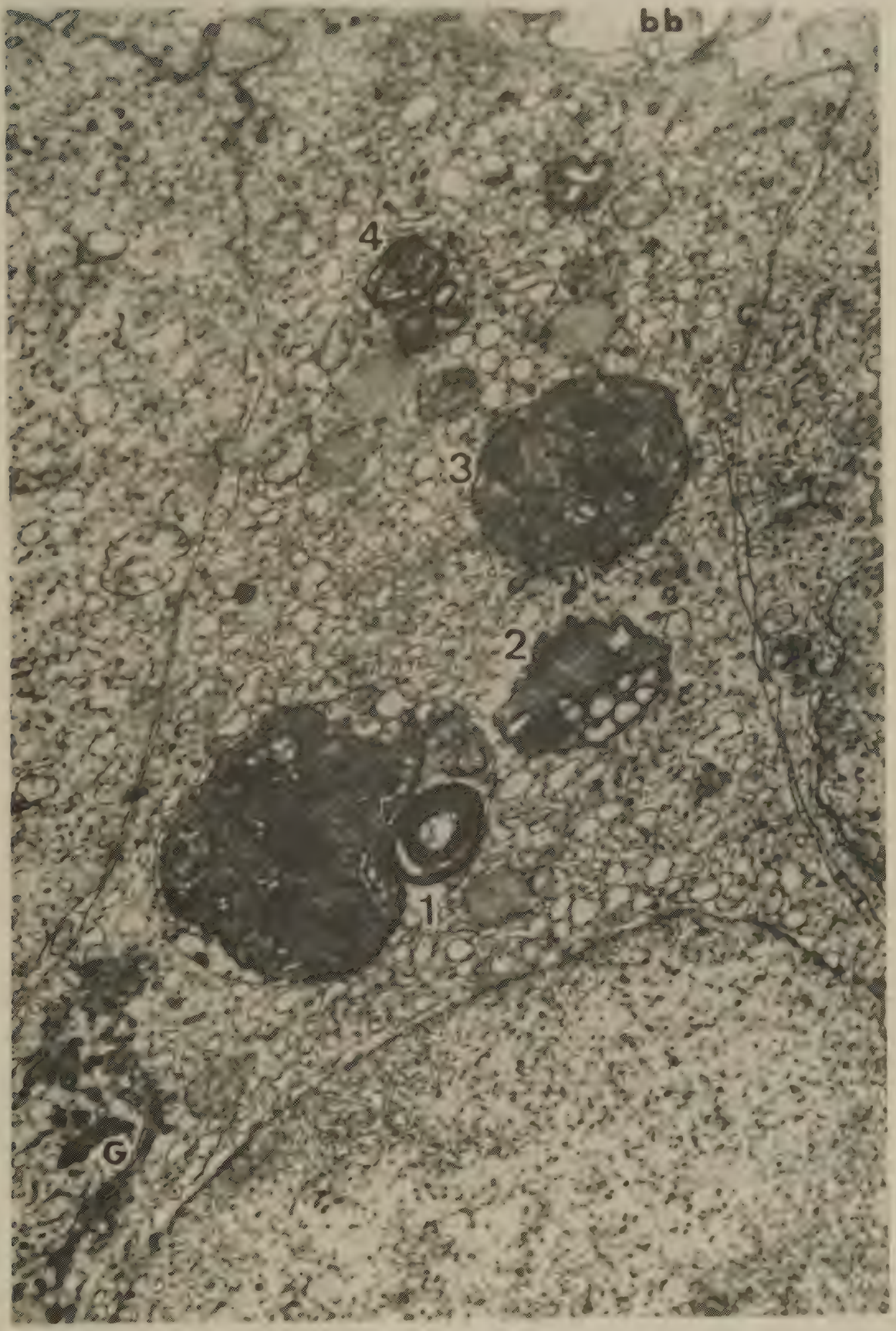



FIG. 10. *Alytes*, stage XX. Highly altered primary epithelium cells. The brush border (bb) has practically disappeared. Golgi cisternae enclose osmiophilic granules (G). The primary autolytic vacuoles are very dense here, and their contents are difficult to recognize (1–3). A primary residual body is also seen (4). ×12,000. (From Hourdry, 1969b.)

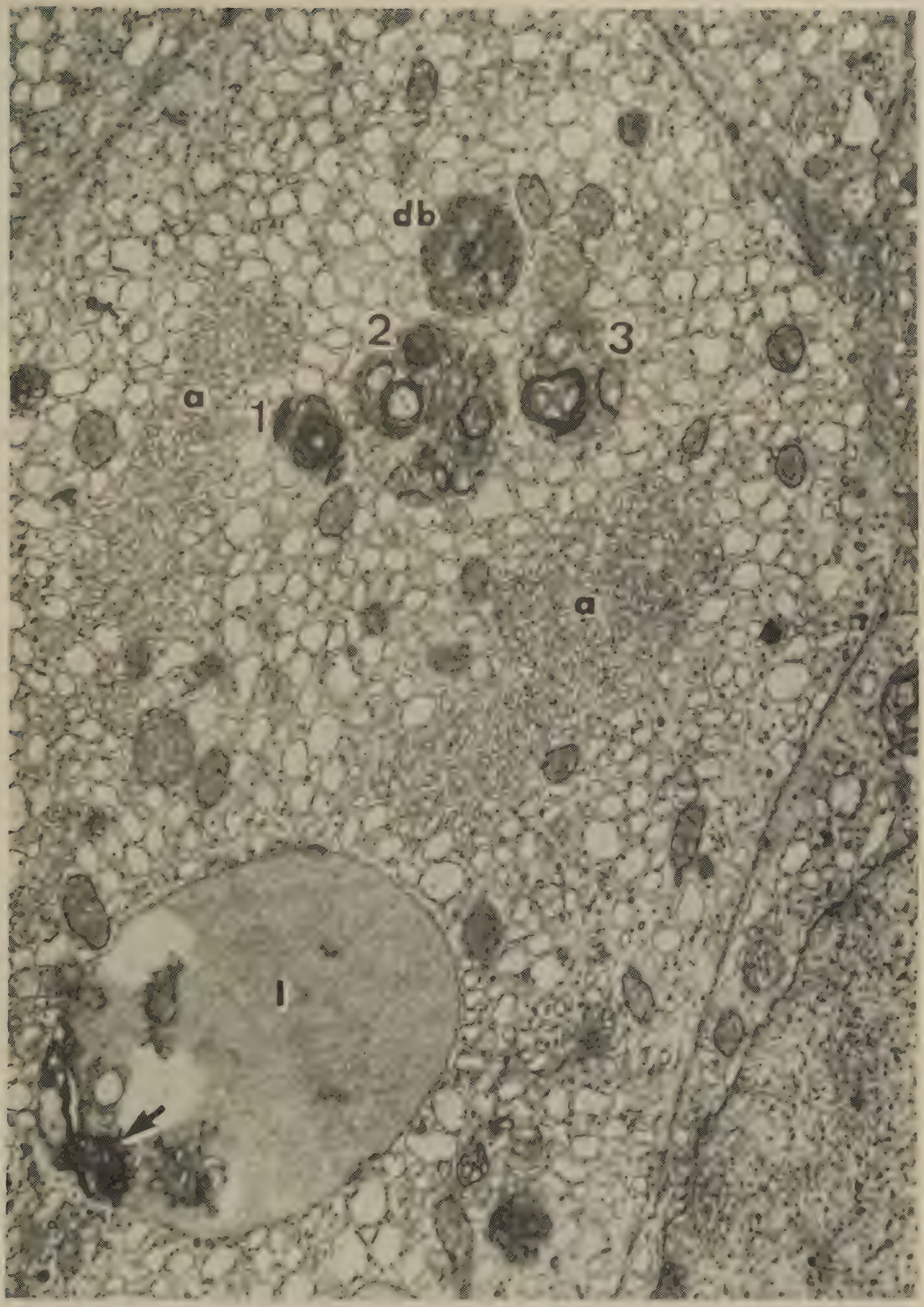



FIG. 11. *Alytes*, stage XIX. Highly altered primary epithelium cell. The endoplasmic reticulum consists of large vesicles or of aggregates of small cisternae (a). At the top, a heterogeneous dense body (db) and three myeloid bodies (1–3) are seen. At the bottom is a lipid droplet (l) containing highly osmiophilic, perhaps membranous, material (arrow). ×12,000. (From Hourdry, 1969b.)

of prometamorphosis, they become quite frequent at the beginning of climax (Figs. 5C and 12). The remnants are then evacuated with the tadpole excreta.

The liberation of discarded material into a natural cavity is not an isolated case. During metamorphosis it has been observed in the intestinal lumen of a dipteran (Misch, 1965); it has also been seen in anurans in the gastric lumen of *Alytes* (Durez, 1963) and in the pancreatic ducts of *Discoglossus* (Beaumont, 1973).

The emission of debris into the hemolymphatic spaces of various

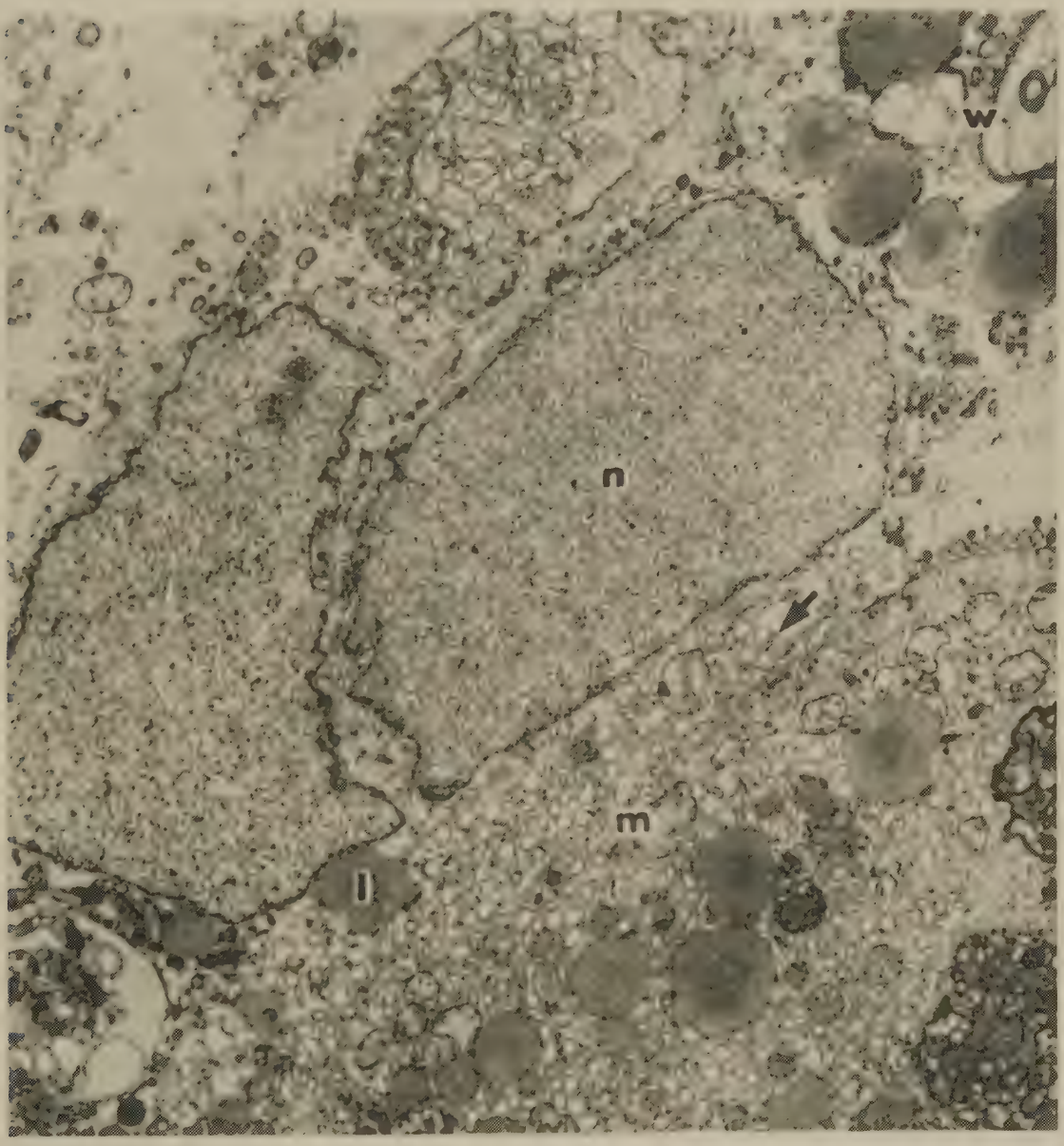



FIG. 12. *Alytes*, stage XX. Degenerated primary epithelium cells liberate their contents into the intestinal lumen after rupture of the apical cell membrane (arrow). Here the evacuated material includes nuclei (n), mitochondria (m), lipid droplets (l), and membrane whorls (w). ×5000. (From Hourdry, 1969b.)

insects has been reported (Scharrer, 1966; Matsuura *et al.*, 1968). In addition, Beaumont (1975) described the passage of necrotized fragments of pancreatic cells into blood capillaries during *Discoglossus* metamorphosis. This type of elimination has not been demonstrated at the level of the gut.

b. *Phagocytosis*. The existence of phagocytic processes in the digestive tube of metamorphosing anurans has been suggested based on light microscope observations. Although Bataillon (1891) stressed only phagocytosis, both Morse (1918) and Kuntz (1924) suggested that cellular autolysis precedes phagocytosis. Janes (1934) emphasized the important role of eosinophils in digestive tube alterations, while Joly (1937) envisaged the resorption of destroyed elements by mononuclear leukocytes. Studying modifications of the intestinal epithelium in *Alytes*, Bernard (1965) also suggested the presence of phagocytic cells.

Electron microscope observation of these cells has shown them to be located in a variety of organs which are sites of natural lytic phenomena including, in metamorphosing anuran larvae, the pancreas (Beaumont, 1975), the gill (Hourdry, 1974a), and the tail (Weber, 1964, 1969; Watanabe and Sasaki, 1974; Fox, 1976). Our observations of intestinal primary epithelium have not confirmed the results reported using light microscopy. No invasion by extraepithelial phagocytic cells was observed (Hourdry, 1972a). The absorption of degenerating regions by a neighboring epithelial cell has nevertheless been suggested at the ultrastructural level by the observation of lysosomes containing apparently exogenous material, such as nuclear fragments (Fig. 13).

## C. Histogenetic Phenomena

Intestinal primary epithelium that degenerates is replaced by secondary epithelium which persists throughout adult life (Hourdry, 1969c).

At the end of prometamorphosis and at the beginning of climax, islets are seen beneath the primary epithelium (Figs. 5B and 14). They are composed of a small number of cells whose embryonic character is affirmed by the high nucleoplasmic ratio and abundance of free ribosomes. During climax, these islets are the site of a "mitotic burst" (Fig. 5C), which leads to transitory pluristratification. At the end of climax, the newly formed tissue folds and forms a simple epithelium (Figs. 5D and 15). The last primary epithelium cells in lysis are pushed out by the new cells and subsequently extruded into the intestinal lumen.

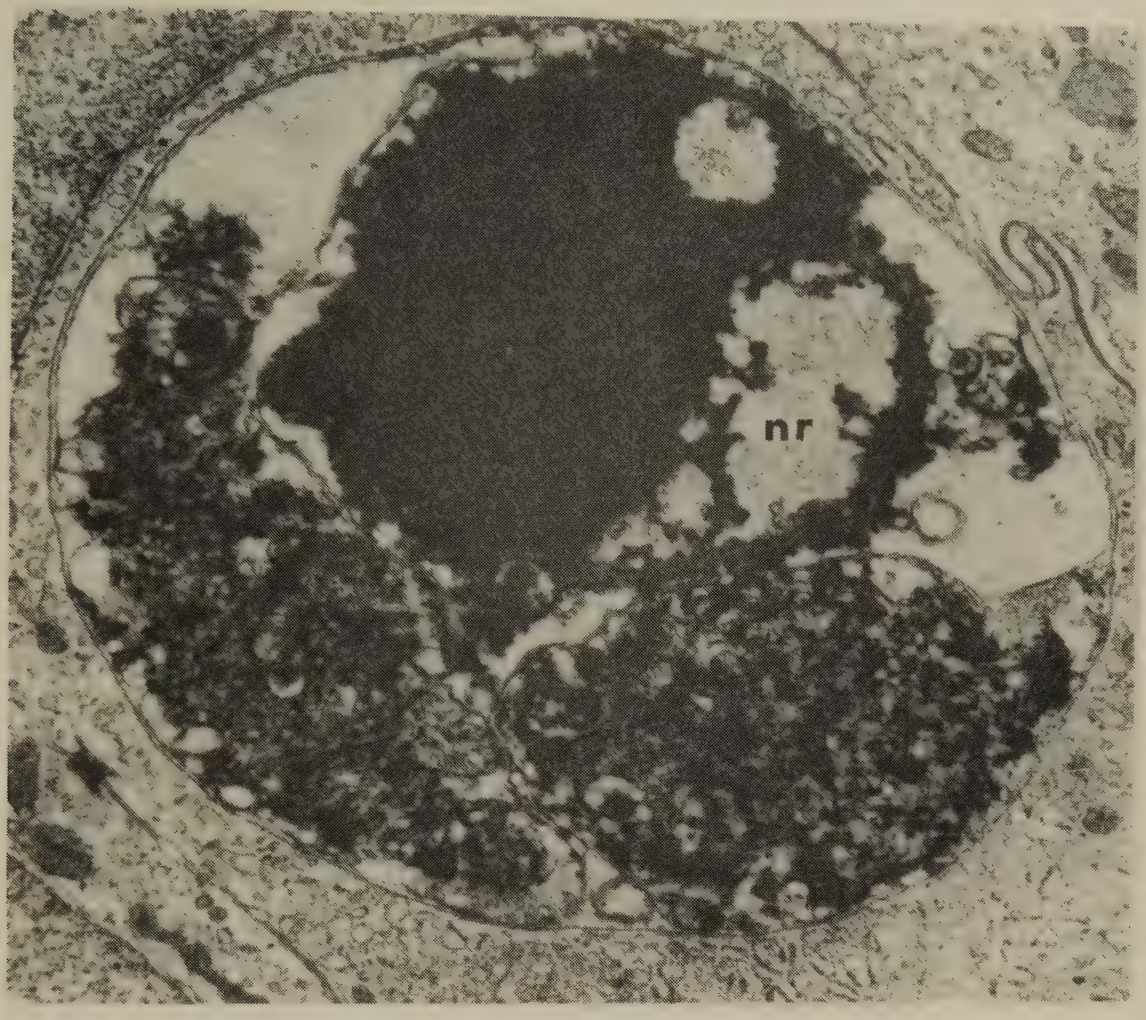



FIG. 13. *Discoglossus*, stage XX. Phagolysosome enclosing nuclear remnants (nr). ×10,000. (From Hourdry, 1971a.)

The rough endoplasmic reticulum of secondary epithelium cells organizes itself in the region of the nucleus into long, parallel cisternae (Fig. 15). The frequency of free ribosomes decreases. Lysosomes are small and infrequent. The fusion of small vesicles with the apical cell membrane permits the expansion of the membrane and the lengthening of microvilli, as observed by Bonneville and Weinstock (1970). According to these investigators, the paranuclear Golgi complex participates in the elaboration of vesicles whose acidic mucopolysaccharide-rich contents permit the formation of a coat on the surface of the microvilli.

The incorporation of tritiated thymidine into nuclear DNA has been studied with autoradiographic techniques (Fig. 16; Hassan and Marinucci, 1966; Dournon and Chibon, 1974; Dauça *et al.*, 1976). The labeling index [(number of labeled nuclei/total number of nuclei) × 100 = percent] of the secondary epithelium has been calcu-

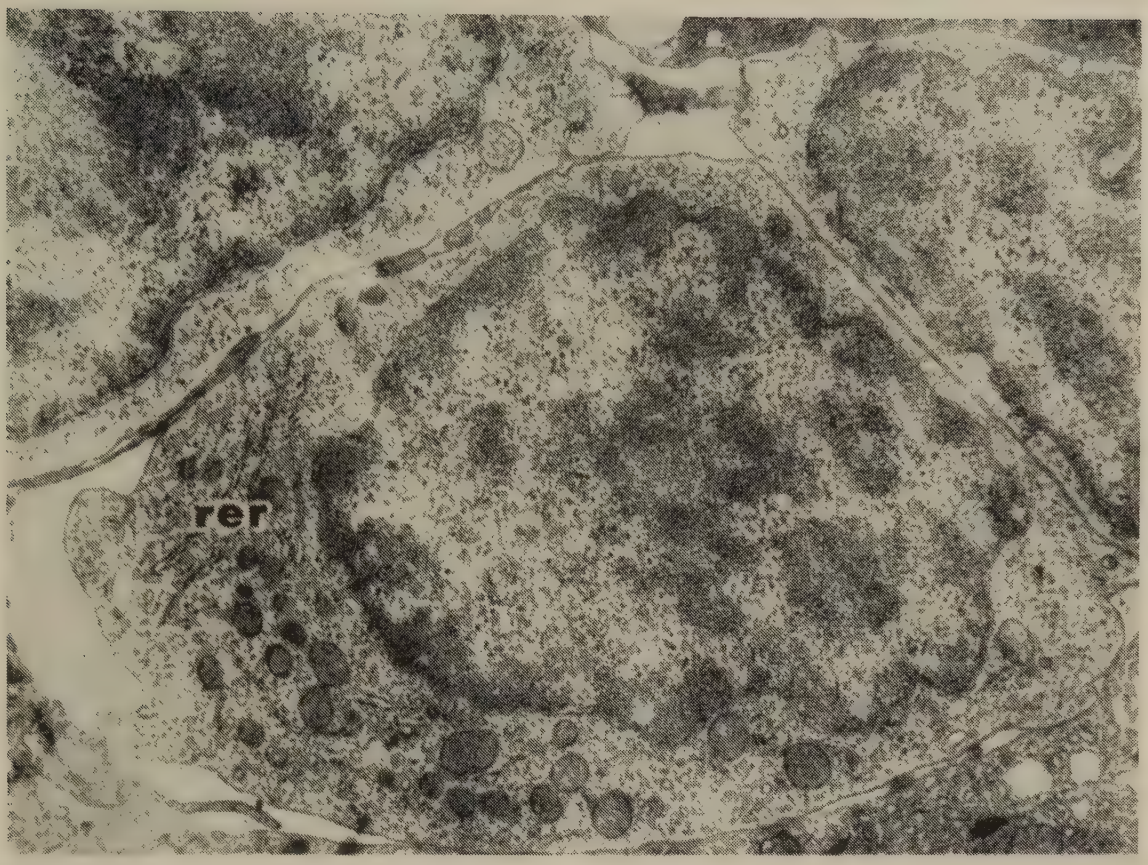
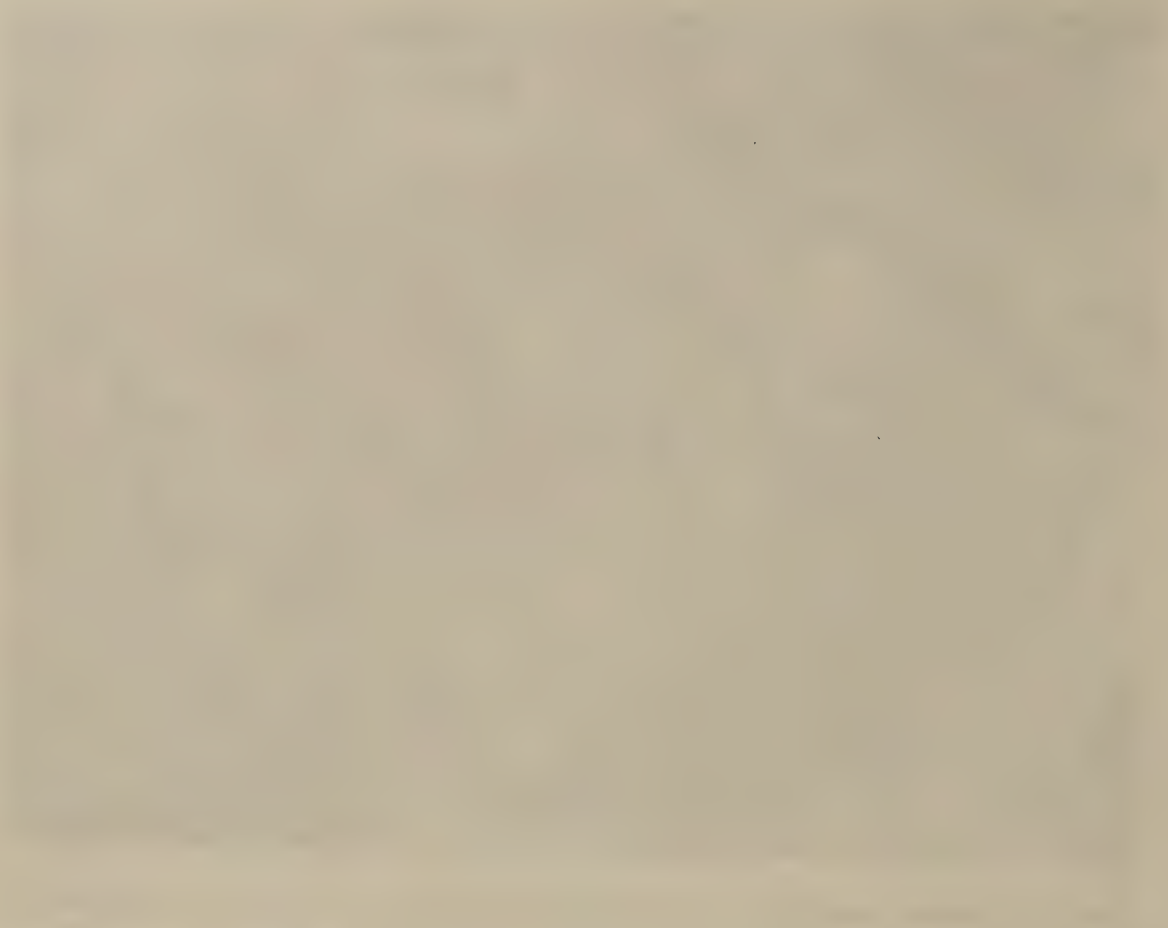



FIG. 14. *Alytes*, stage XIX. Embryonic cells at the base of the primary epithelium. The nucleus is surrounded by a thin cytoplasmic band rich in free ribosomes. Several cisternae of the rough endoplasmic reticulum are seen (rer). ×7000.

lated and allows the proliferation rate of the tissue to be determined. In *Alytes* larvae a labeling index greater than 40% is obtained at the moment of climax, which decreases during postclimax (Dauça *et al.*, 1976). In a parallel fashion, the specific activity of DNA, expressed as counts per minute per microgram, increases 10-fold from prometamorphosis to climax (M. Dauça and J. Hourdry, unpublished observations). Several workers have suggested a proliferation of embryonic stem cells from certain regions, in particular the point of reflection of the gut (Delsol and Zervudacki, 1963; Dournon and Chibon, 1974). We have not, however, been able to confirm this hypothesis, since the labeling index of secondary epithelium does not vary significantly from one intestinal region to the other (M. Dauça and J. Hourdry, unpublished observations).

The replacement of degenerating primary epithelium by secondary epithelium is summarized in Fig. 17.



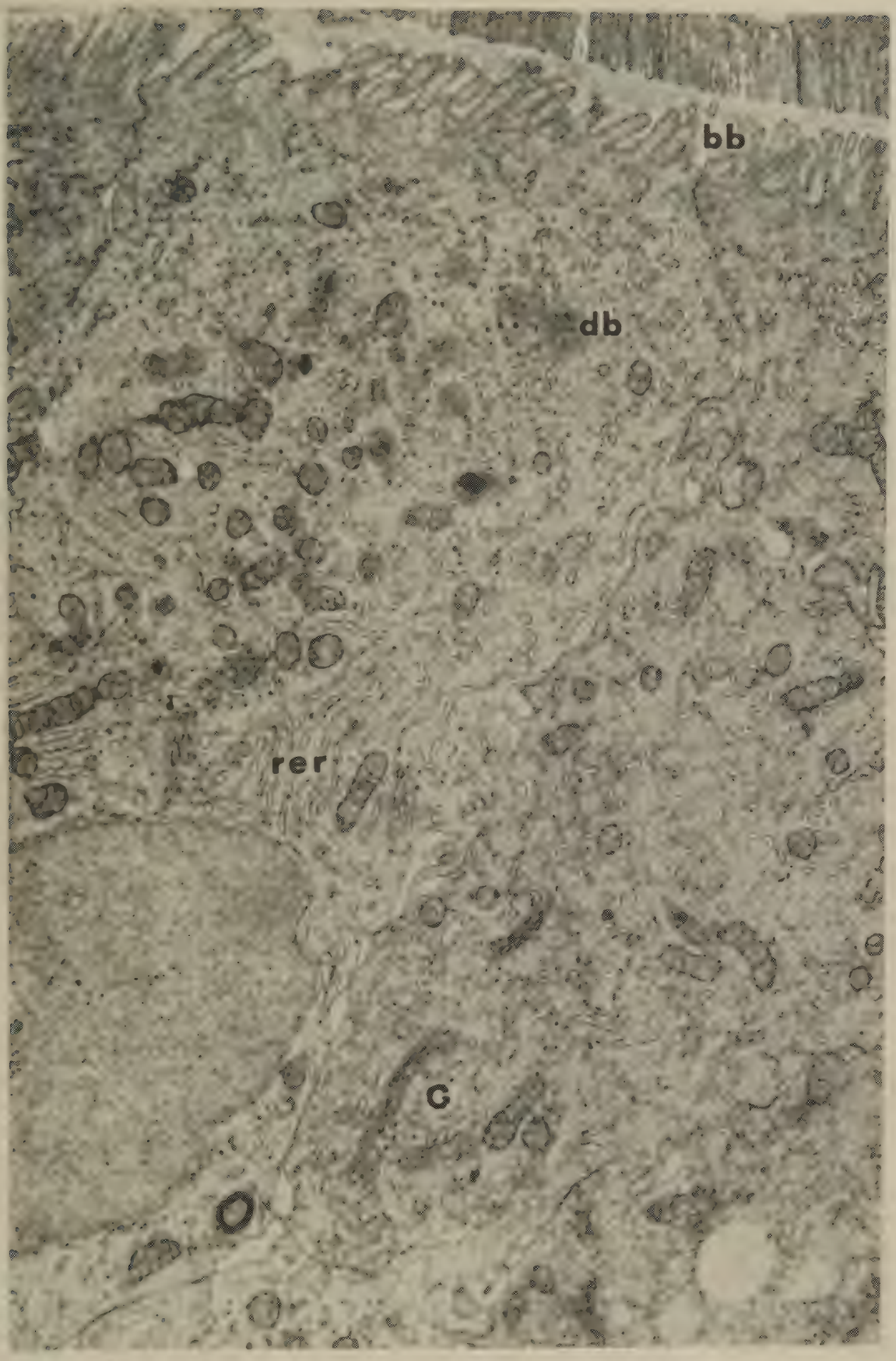



FIG. 15. *Alytes*, stage XXIII. Secondary epithelial cells in place. The rough endoplasmic reticulum is organized in parallel cisternae (rer) near the nucleus. bb, Brush border; db, Dense body; G, Golgi complex. ×14,000. (From Hourdry, 1969c.)

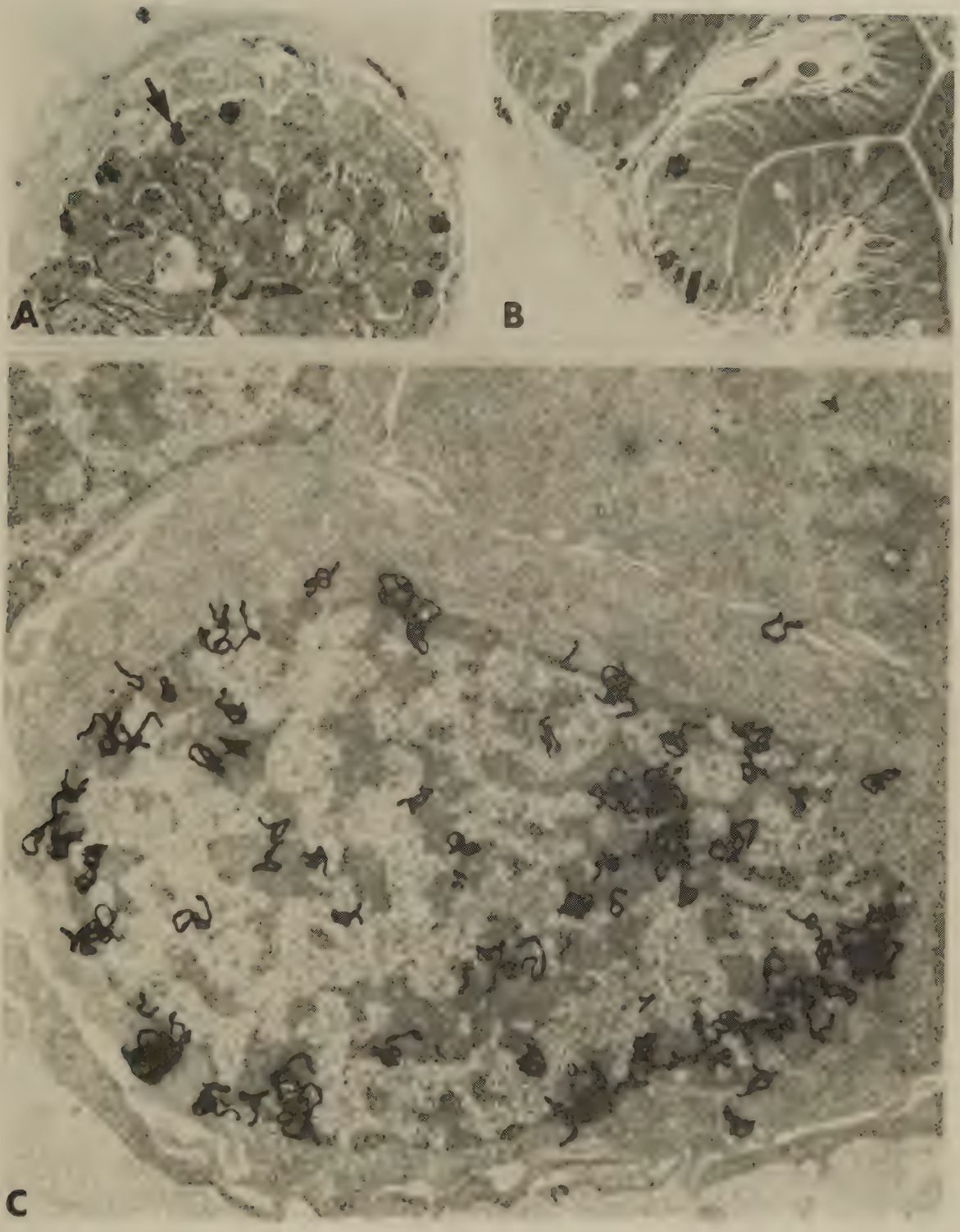
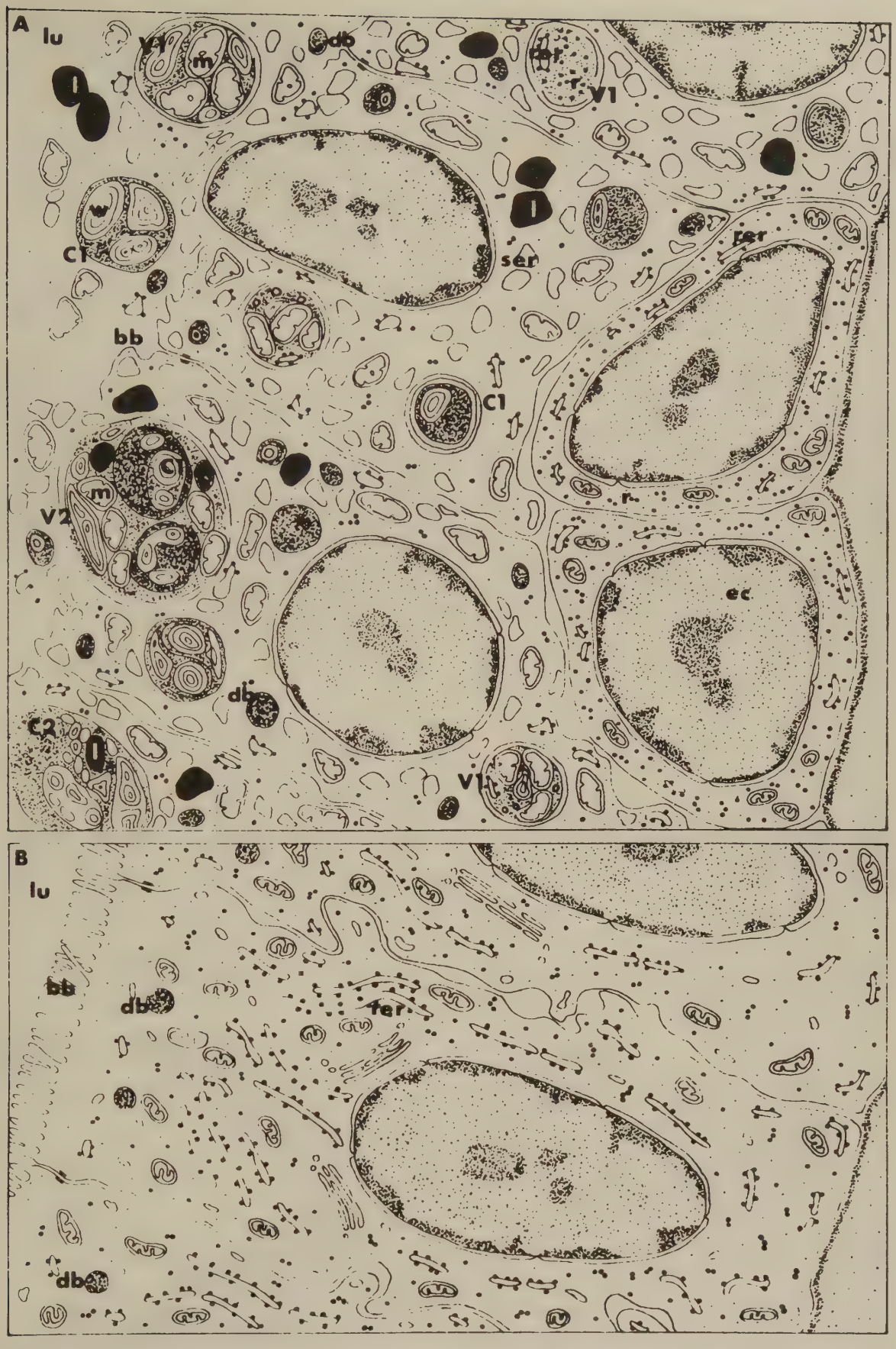



FIG. 16. Intestinal epithelium of *Discoglossus* larvae which received 6 $\mu$Ci of tritiated thymidine. (A and B) Semithin sections. (A) Stage XX. Several labeled nuclei are seen in the embryonic islets (arrow). ×250. (B) Stage XXIII. Labeled nuclei are found at the base of the microvilli of the secondary epithelium.×300. (C) Stage XX. Silver grains are present on one of the nuclei of this islet. ×10,000.

FIG. 17. Intestinal epithelium undergoing transformations. (A) Epithelium at the beginning of climax. The content of the primary epithelium cells is evacuated into the intestinal lumen (lu). At the right, two embryonic cells (ec) can be seen. (B) Secondary epithelium at the end of climax. bb, Brush border; C1, primary residual body; C2,




secondary residual body; db, small dense body; l, lipid droplet; m, mitochondrion; r, ribosome; rer, rough endoplasmic reticulum; ser, smooth endoplasmic reticulum; V1, primary autolytic vacuole; V2, secondary autolytic vacuole; w, membrane whorl. (From Hourdry, 1973a.)

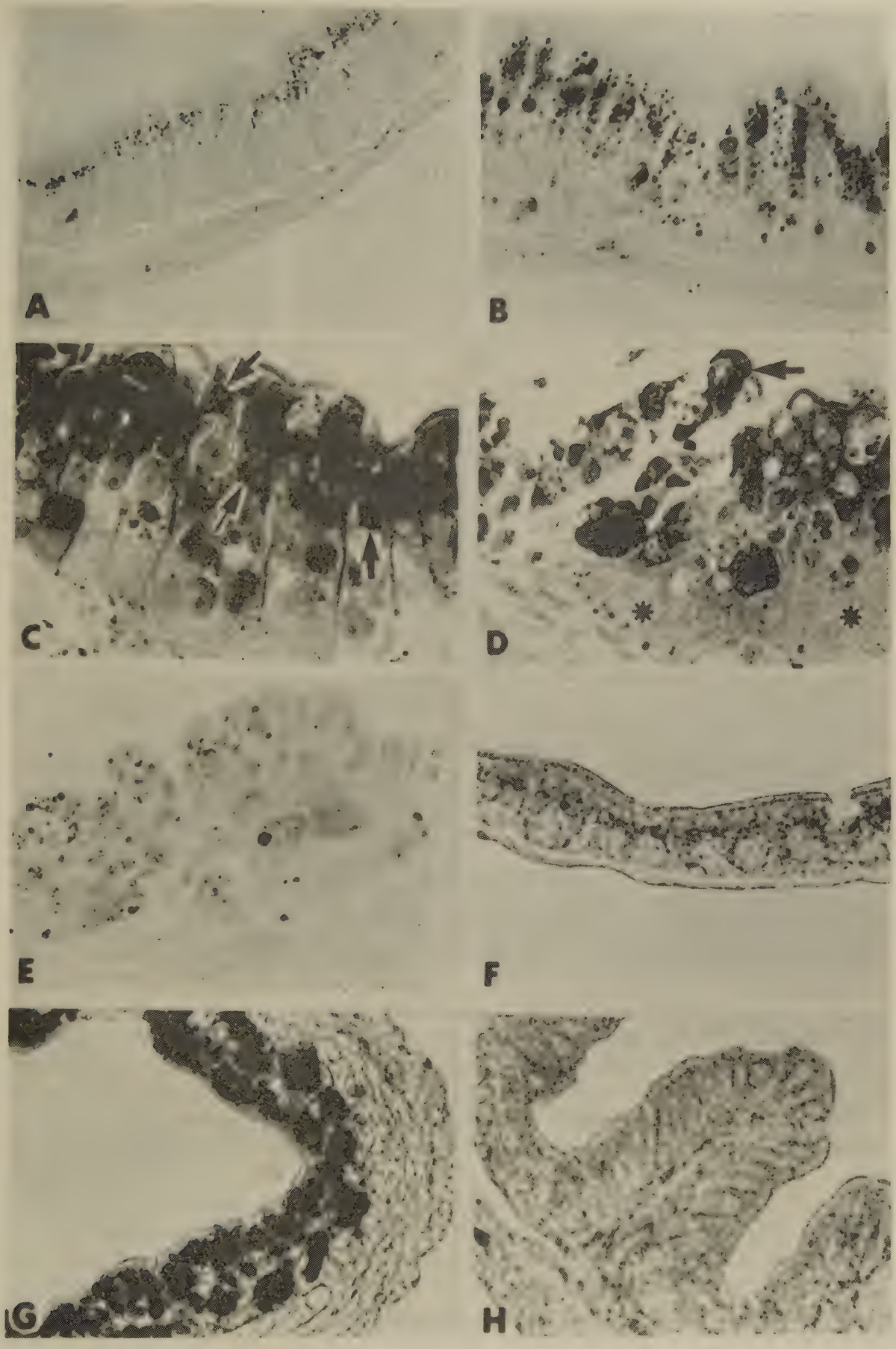
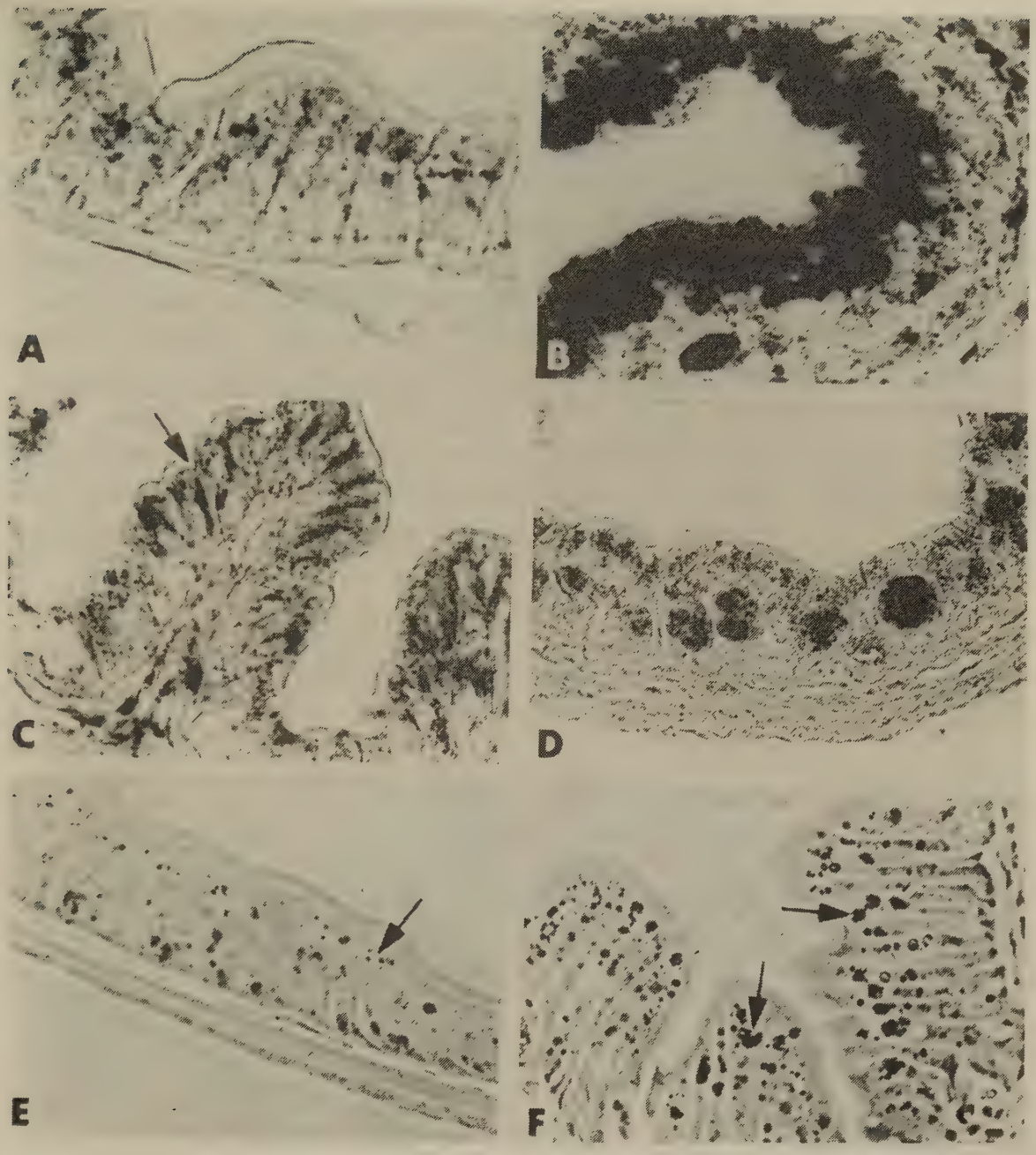



FIG. 18. Acid phosphatase activity in the intestinal epithelium of *Discoglossus* larvae. (A–E) Semithin sections. (A–D) Activity is demonstrated in the primary epithelium by a lead sulfide precipitate. (A) Stage XIII. ×300. (B) Stage XVIII. ×350. (C) Stage XIX. Activity is high, as observed in large lysosomes (thick arrows), as well as




diffuse, as seen in the hyaloplasm (thin arrow) ×400. (D) Stage XXI. A portion of the primary epithelium is liberated into the intestinal lumen (arrow). Two islets of embryonic cells show no enzyme activity (asterisks). ×250. (E) Stage XXI. Inhibition of activity by sodium fluoride ×300. (F–H) Frozen sections. (F and G) Activity is visualized in the primary epithelium by an azo dye. (F) Stage XIII. ×250. (G) Stage XIX. ×300. (H) Stage XXIII. The secondary epithelium shows no reaction. ×300. (From Hourdry, 1971b.)

FIG. 19. (A–F) Intestinal epithelium of *Discoglossus* larvae. (A–C) The activity of *N*-acetyl-β-glucosaminidase is detected in frozen sections by the deposit of an azo dye. (A) Stage IV. ×300. (B) Stage XX. Intense activity is seen at the level of the primary epithelium. ×300. (C) Stage XXIII. The reaction is slightly positive in the secondary epithelium (arrow). ×300. (D) Stage XX-XXI. β-Glucuronidase activity is visualized in frozen sections of the primary epithelium by an azo dye deposit. ×300. (E and F) Aryl sulfatase activity is visualized in semithin sections of the primary epithelium by a deposit of barium sulfate, which is more refringent in phase-contrast microscopy (arrows). (E) Stage XIV. ×300. (F) Stage XIX. ×300. (From Hourdry, 1971b.)

## D. Lysosomal Hydrolases

Many lytic phenomena are associated with the activity of lysosomal enzymes which are capable of hydrolyzing various chemical components present in the cell (de Duve, 1963). As a result of its great stability and the ease with which it is demonstrated in sections, acid phosphatase activity is the most characteristic of lysosomes (de Duve, 1963; Novikoff, 1963). It has been sought in the intestinal epithelial cells of the larvae of *Rana esculenta* (Hassan and Marinucci, 1966) and of *D. pictus* (Hourdry, 1971b,c). In the latter species, the activity of four other lysosomal hydrolases has also been studied by histochemical techniques: aryl sulfatase, thiolacetate esterase, β-glucuronidase, and *N*-acetyl-β-glusosaminidase (Hourdry, 1971b).

### 1. *Light Microscopy*

Hydrolytic activity has been visualized in semithin and frozen sections (Hassan and Marinucci, 1966; Hourdry, 1971b). All show a

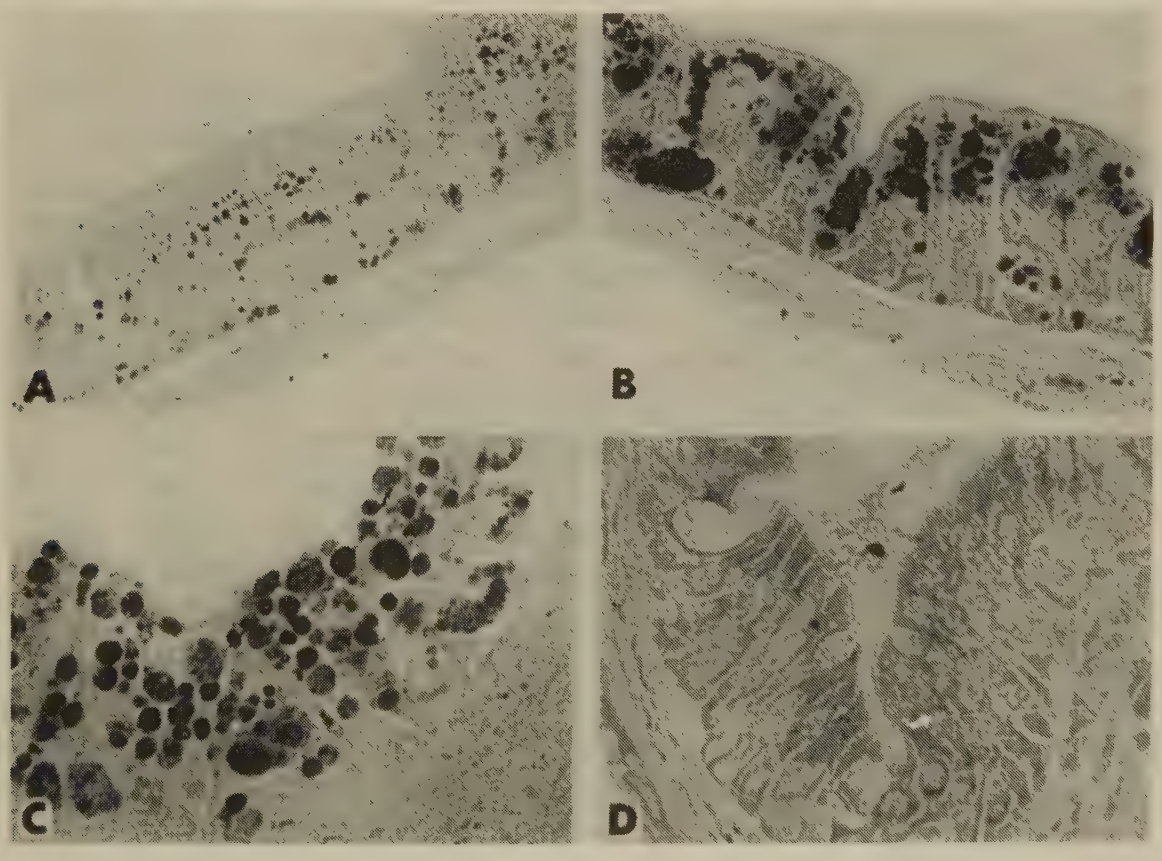



FIG. 20. Thiolacetate esterase activity in the intestinal epithelium of *Discoglossus* larvae. (A–D) Semithin sections. (A–C) The activity is detected in the primary epithelium by a deposit of lead sulfide. (A) Stage XIV. ×300. (B) Stage XIX. ×300. (C) Stage XX. ×300. (D) Stage XXIII. No enzyme activity is apparent in the secondary epithelium. ×350. (From Hourdry, 1971b.)

TABLE I
VARIATIONS OF ENZYME ACTIVITY IN THE INTESTINAL PRIMARY EPITHELIUM OF *Discoglossus* TADPOLES AS A FUNCTION OF THE DEVELOPMENTAL STAGE[a,b]

| Enzymes | Pre-metamorphosis | Prometamorphosis | | | Climax | | |
|---|---|---|---|---|---|---|---|
| | Before XII | XII to XVII | XVIII | XIX | XX | XXI | After XXI |
| Acid phosphatase | ± | ± | + | ++ | ++ | ++ | − |
| Aryl sulfatase | ± | ± | ± | + | ++ | + | − |
| Thiolacetate-esterase | ± | ± | + | ++ | ++ | ++ | − |
| β-Glucuronidase | − | − | − | − | ± | ± | − |
| *N*-Acetyl-β-glucosaminidase | ± | + | + | ++ | +++ | ++ | ± |

[a] Taylor and Kollros (1946).
[b] Strongly positive, +++; positive, ++; weakly positive, +; negative, −.

similar pattern of variation in the primary epithelium (Figs. 18–20). Initially limited to several small grains of the supranuclear space, enzyme activity is demonstrated in numerous, voluminous lysosomes at the end of prometamorphosis and at the beginning of climax. Diffuse activity is occasionally observed, beginning at the end of prometamorphosis. The intensity of the reaction is a function of the particular hydrolase, being low for β-glucuronidase at the beginning of climax but becoming very high for *N*-acetyl-β-glucosaminidase.

The enzyme activity of the secondary replacement epithelium is almost always negative.

These results are summarized in Table I.

The total activity of several lysosomal hydrolases (acid phosphatase, *N*-acetyl-β-glucosaminidase, β-glucuronidase, aryl sulfatase, and cathepsin) has been calculated for the intestines of various anuran larvae (Botte and Buonanno, 1962; Hourdry, 1974b). Each activity increases significantly at the end of prometamorphosis and at the beginning of climax, at the moment of degeneration of the primary epithelium. The preceding histochemical observations are thus confirmed by these results.

### 2. *Electron Microscopy*

The hydrolytic activity most intensely studied in thin sections is that of acid phosphatase (Hourdry, 1971c). Before degeneration of the pri-

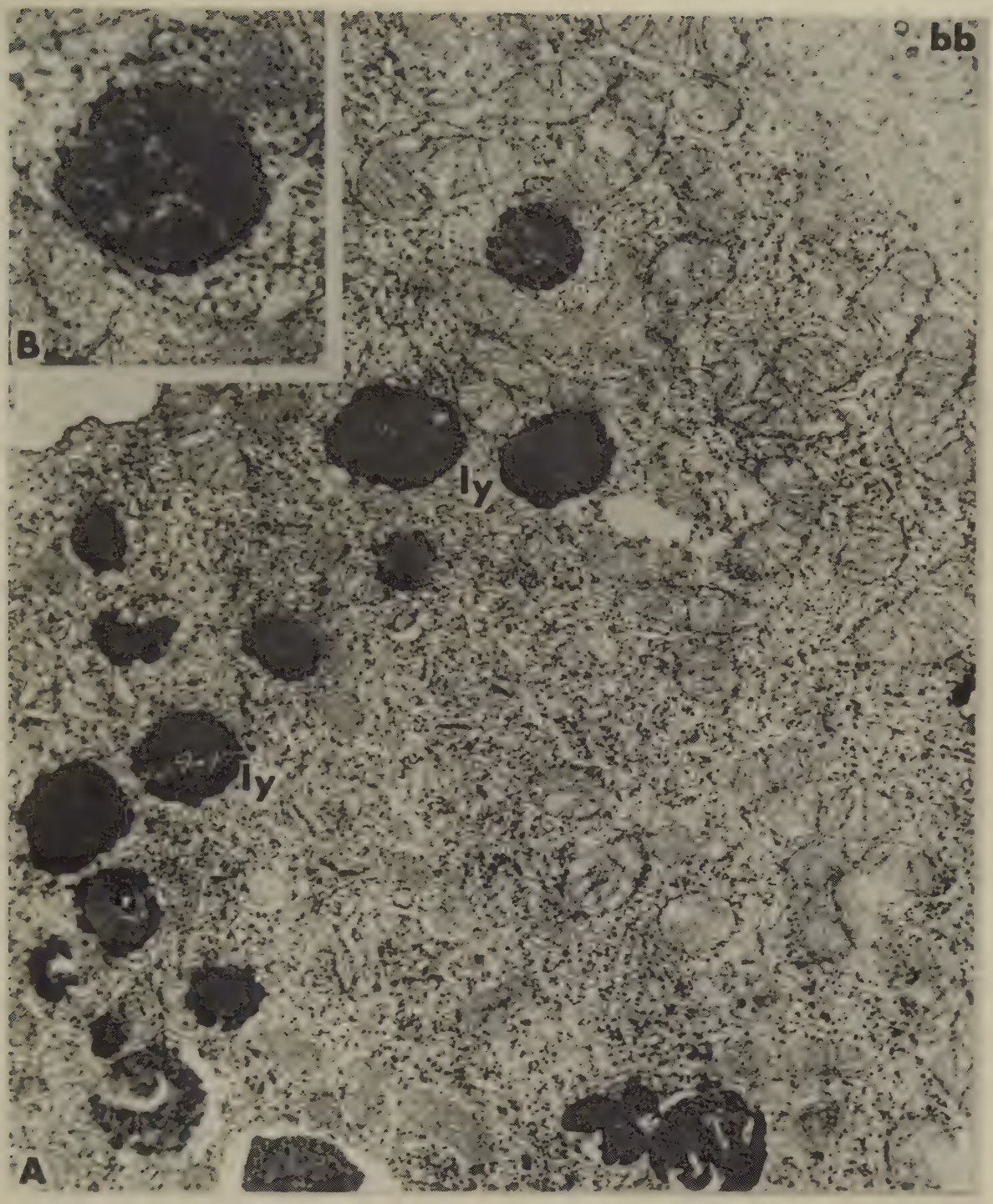



FIG. 21. (A) *Discoglossus*, stage XIII. Acid phosphatase activity is visualized by a lead phosphate deposit. It is seen in the small supranuclear lysosomes (ly) of this primary epithelium cell. At top right, an outcropping of brush border (bb) is seen. ×25,500. (B) Detail. ×56,000. (From Hourdry, 1974c.)

mary epithelium, this activity is confined to the small lysosomes of the supranuclear space (Fig. 21). These lysosomes become more numerous at the end of prometamorphosis, and their acid phosphatase activity diffuses into the hyaloplasm (Fig. 22). Some of them are virgin lysosomes; others are small residual bodies which can contribute their enzyme load to a new degenerative process.

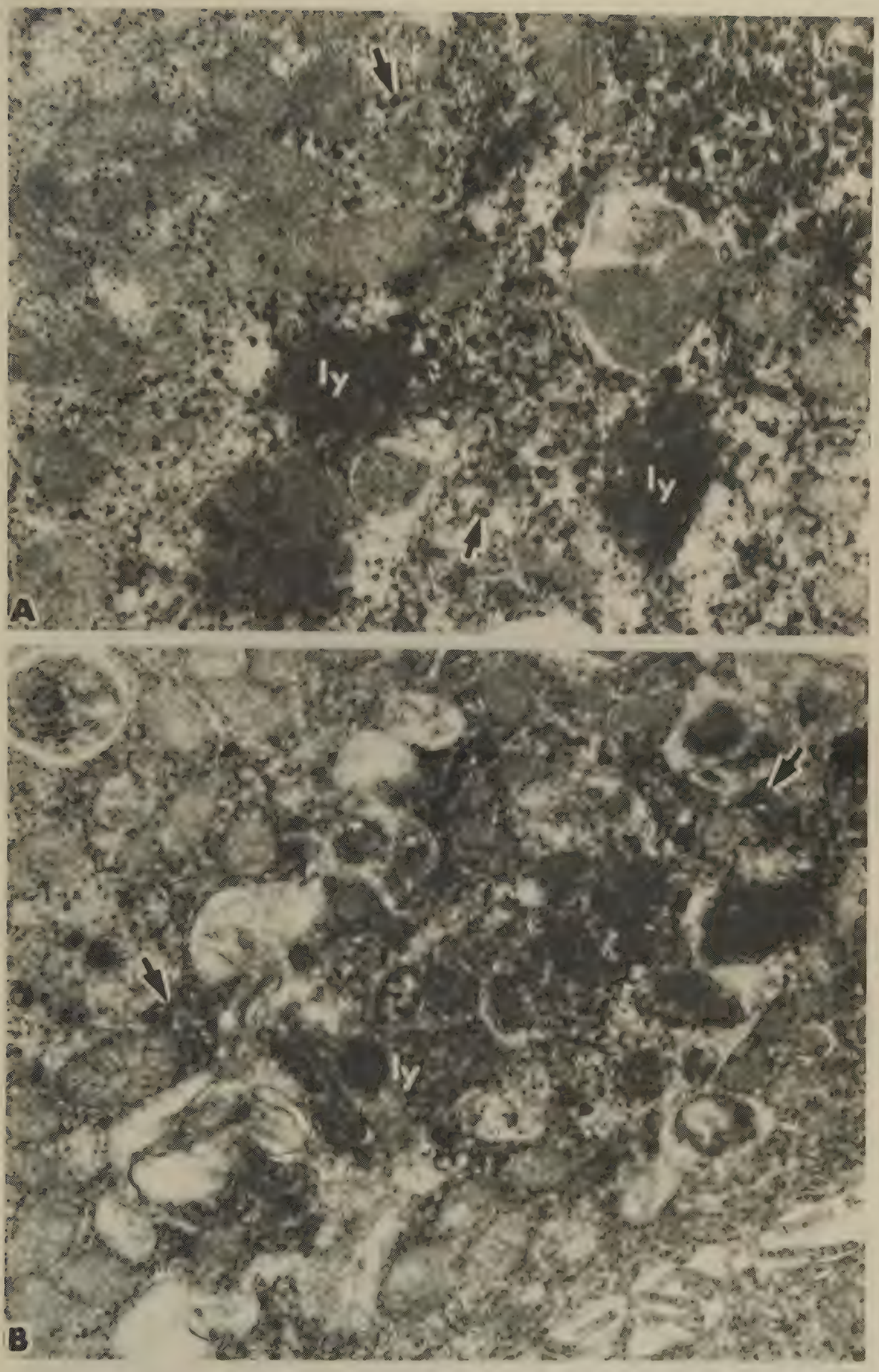
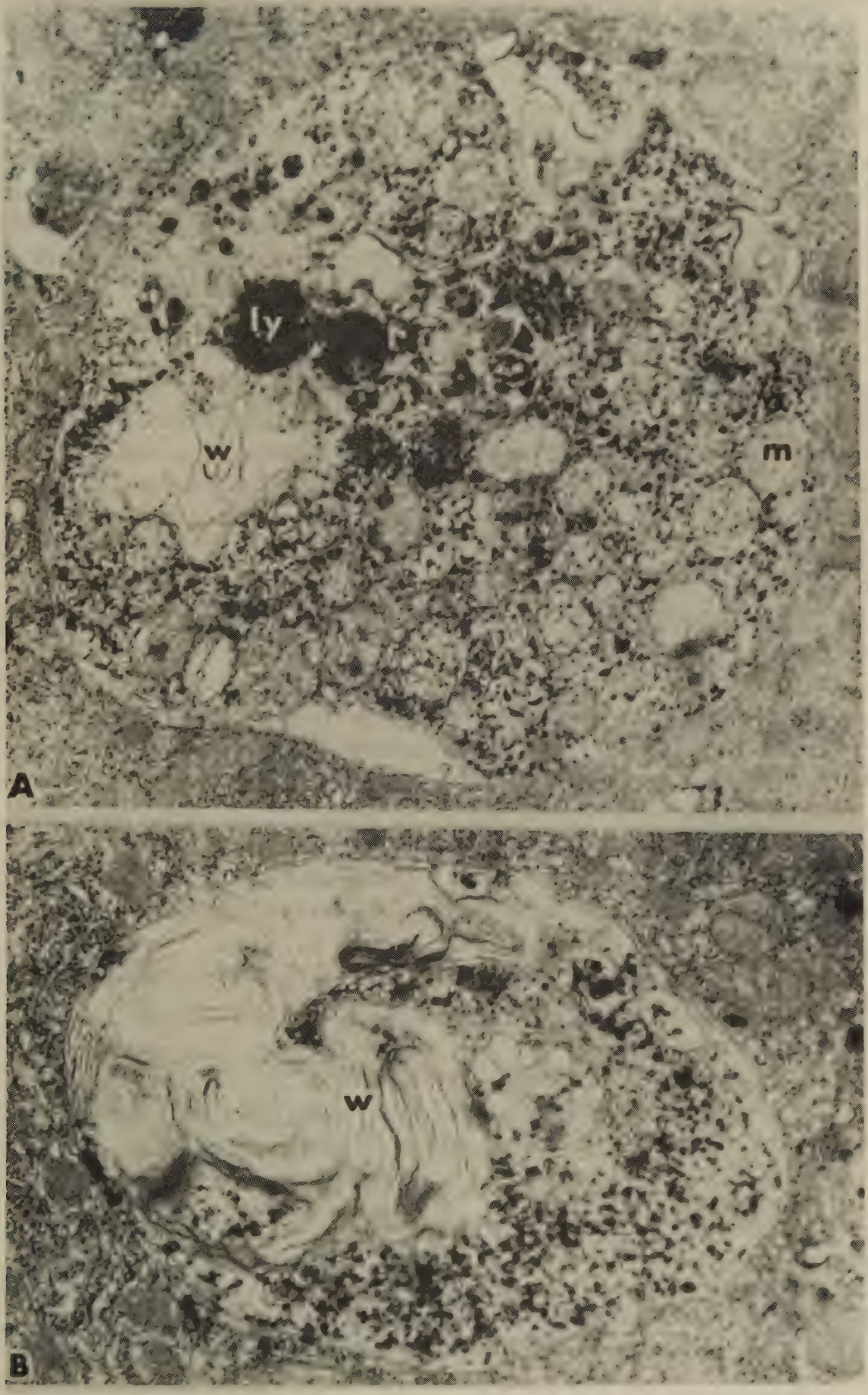



FIG. 22. *Discoglossus*, stage XIX. Primary epithelium cells; acid phosphatase activity is detected both in small lysosomes (ly) and in the surrounding hyaloplasm (arrows). (A) ×21,000. (B) ×22,000. (From Hourdry, 1971c.)


ly
w
m
A
w
B

The activity of intestinal soluble acid phosphatase has been measured in *Discoglossus* tadpoles. The increase in this activity coincides with the appearance of reaction product in the hyaloplasm (Hourdry, 1974b).

The observed diffusion results from activation of the enzyme-accumulating lysosomes discussed above; that is, their membrane is rendered permeable or actually ruptures (de Duve, 1963). Lysosomal activation, with attendant liberation of necrotizing hydrolases, is generally believed to occur during natural autolytic processes (de Duve, 1963; de Duve and Wattiaux, 1966) and has been proven by histochemical and biochemical arguments. Thus the existence of diffuse enzyme activity in the hyaloplasm is observed very often during involutive phenomena (Misch, 1965; Lockshin and Williams, 1965; Osinchak, 1966; Schin and Clever, 1968). Similarly, an increase in the soluble activity of several hydrolases has been reported in various regressing organs (Scheib, 1963; Russo-Caia and Hassan, 1965; Salzgeber and Weber, 1966; Broaddus-Patel, 1970; Perriard, 1971; Robinson, 1972; Buser, 1972).

The mechanism permitting the diffusion of these hydrolases is not well known. Tappel *et al.* (1963) suggested lipid peroxidation at the level of the lysosomal membrane where, according to Gahan (1967), the lipoprotein bond is attacked.

Acid phosphatase activity is also visualized in primary and secondary autolytic vacuoles and in their residual forms (Fig. 23). The various lysosomes are equivalent to the large grains seen with light microscopy.

A slight deposit of reaction product (lead phosphate) can be detected in the Golgi saccules, especially at their extremities. Also exhibiting activity are several vesicles located near the dictyosomes.

### 3. *Fate of Lysosomal Enzymes*

It is not very probable that lysosomal enzymes are inactivated and subsequently recovered by the animal; rather, they are more likely eliminated from the larva through the intestinal lumen, as has tentatively been proven by Varute and Sawant (1972). These investigators observed a maximum of $\beta$-glucuronidase activity in tadpole excreta at the moment of metamorphosis.

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FIG. 23. *Discoglossus*, stage XX. Acid phosphatase activity in primary epithelium cells. (A) Autolytic vacuole where mitochondria (m), membrane whorls (w), and enzyme-accumulating lysosomes (ly) can be seen. ×22,500. (B) Residual body rich in membrane whorls (w). ×27,000. (From Hourdry, 1974c.)

### 4. *Onset of Cellular Degeneration*

The lysosomal hydrolases liberated into the hyaloplasm initiate the degenerative phenomena observed in intestinal primary epithelium. The temptation to generalize must be resisted, since cytoplasmic alterations can in fact appear in other tissues without the involvement of these enzymes; this point of view is shared by several workers (Weber, 1969; Radford and Misch, 1971; Schiaffino and Hanzlíková, 1972b). The accumulation of degradation products would secondarily induce the appearance of lysosomes; thus the increase in lysosomal hydrolase activity would no longer be the cause, but rather the effect or the symptom, of tissue involution (Weber, 1969; Helminen and Ericsson, 1970).

## E. Autolytic Vacuoles and Their Residual Forms as Part of the Alteration of Intestinal Primary Epithelium

### 1. *Origin of the Vacuolar Enzymes*

Three origins are possible for the vacuolar enzymes:

a. *Cisternae of the Smooth Endoplasmic Reticulum.* By surrounding certain vacuoles, these cisternae become a potential source of hydrolases following rupture of the internal membrane. Nevertheless, the amount of enzyme contained in the cisternae is certainly too small to provoke sustained intravacuolar degradation. No acid phosphatase activity was demonstrated in these cisternae (Hourdry, 1971c).

b. *Golgi Vesicles.* Acid phosphatase activity is occasionally observed in these organelles (Hourdry, 1971c). Their presence in the vacuoles suggests a supply of hydrolases (Fig. 6B), which is, however, itself certainly insufficient to induce significant degeneration of the segregated material.

c. *Enzyme-Accumulating Lysosomes.* These lysosomes are larger than the Golgi vesicles but do not exceed 1 $\mu$m in diameter; they constitute the principal source of enzymes for autolytic vacuoles (de Duve and Wattiaux, 1966; Deter, 1971), either fusing with them or penetrating them. Our observations suggest this lysosomal origin: It was observed in several micrographs taken during epithelial alteration that the acid phosphatase activity of accumulating lysosomes had diffused into the interior of a vacuole (Hourdry, 1971c; Fig. 23A). Some enzyme-accumulating lysosomes are virgin lysosomes; others are small, preexisting residual bodies.

The Golgi apparatus is generally more developed in intestinal primary epithelium cells at the end of prometamorphosis. A relation be-

tween the Golgi saccules and virgin lysosomes has been established in some cases.

### 2. *Evolution of the Contents of Vacuoles*

The residual bodies observed in primary epithelium cells undergoing lysis contain myeloid whorls and dense patches, resulting from the accumulation of material that has not been hydrolyzed by the lysosomal enzymes. The myeloid figures represent undigested phospholipids deposited in membrane whorls in the intravacuolar aqueous phase. Although several phospholipases have been demonstrated in the lysosomes of liver, kidney, and brain (Mellors and Tappel, 1967; Weinreb *et al.*, 1968; Franson *et al.*, 1971), they appear to be only slightly active. The dense patches could contain lipofuscin; since pigments appear during the successive steps of lipoid autooxidation.

Does the animal reutilize some metabolites which appear in the lysosomes and diffuse into the degenerating cells? Although this possibility remains to be verified, it would be consistent with the energy needs of a larva that not only undergoes a significant transformation but also no longer ingests.

### 3. *Interpretation of Autolytic Vacuoles and Their Residual Forms*

Several interpretations have been given for autolytic vacuoles (de Duve and Wattiaux, 1966). In particular, they may protect the cell from generalized necrosis by isolating the altered regions (Napolitano, 1963). Ashford and Porter (1962) have thus interpreted them as "physiological autolytic foci" automatically surrounded by a protective membrane.

In intestinal primary epithelium cells at the end of prometamorphosis, autolytic vacuoles may also represent a defensive reaction to generalized degeneration (Hourdry, 1972a). The hydrolases in the small preexisting lysosomes may diffuse into the hyaloplasm of the epithelial cells, inducing the development of autolytic areas. The cells react to this aggression by isolating the altered regions inside primary autolytic vacuoles which become progressively transformed into primary residual bodies.

The extension of enzyme diffusion leads to a second defensive reaction, with the appearance of secondary autolytic vacuoles which engulf the lysosomes. This barrier in turn seems insufficient, since enzyme activity is still detected in the hyaloplasm. Finally, the entire cell is attacked by the hydrolases, and its fragments liberated into the intestinal lumen.

The membrane does not appear spontaneously here, but rather is formed from smooth endoplasmic reticulum.

### F. Conclusion

The intestinal epithelium of metamorphosing anurans is the site of histolytic and histogenetic processes. Intestinal primary epithelium that degenerates is replaced by secondary epithelium generated by the multiplication of embryonic stem cells.

A double mechanism is responsible for the destruction of primary epithelium cells. Initially, the number of small lysosomes increases, beginning in the middle of prometamorphosis; in parallel, the activity of several hydrolases associated with these organelles rises in a characteristic fashion. Subsequently, the lysosomes are activated, and their hydrolases liberated into the hyaloplasm.

Autolytic vacuoles and their residual forms appear in the epithelial cells; by isolating the most highly altered areas, they act as a form of cellular defense against the progression of degeneration.

Necrotized material is extruded into the intestinal lumen. No invasion by extraepithelial phagocytic cells is observed.

Villous secondary epithelium, which persists in the adult, develops under primary epithelium undergoing lysis.

A herbivore before metamorphosis, the animal becomes a carnivore afterward. Ingestion ceases during climax. The primary epithelium cells are at this time too altered to play a part in absorption, and the secondary epithelium is not yet in place.

## III. The Intestinal Epithelium of Larvae following Experimental Modification of the Level of Circulating Thyroid Hormones

As a result of the research of Gudernatsch (1912), anuran metamorphosis is attributed to the activity of thyroid hormones. The majority of metamorphic events take place when the plasma level of these hormones passes through a maximum, reaching a concentration of $10^{-7}$ gm/ml (Etkin, 1968).

The effects of these hormones on the intestinal epithelium have been demonstrated by altering their concentration in the plasma. Tadpoles were subjected to one of the following treatments: (1) immersion in a $T_4$ solution (Hourdry, 1971d); (2) treatment with an antithyroid agent, potassium perchlorate (Hourdry, 1973b); or (3) thyroidectomy or hypophysectomy (Hourdry, 1972b).

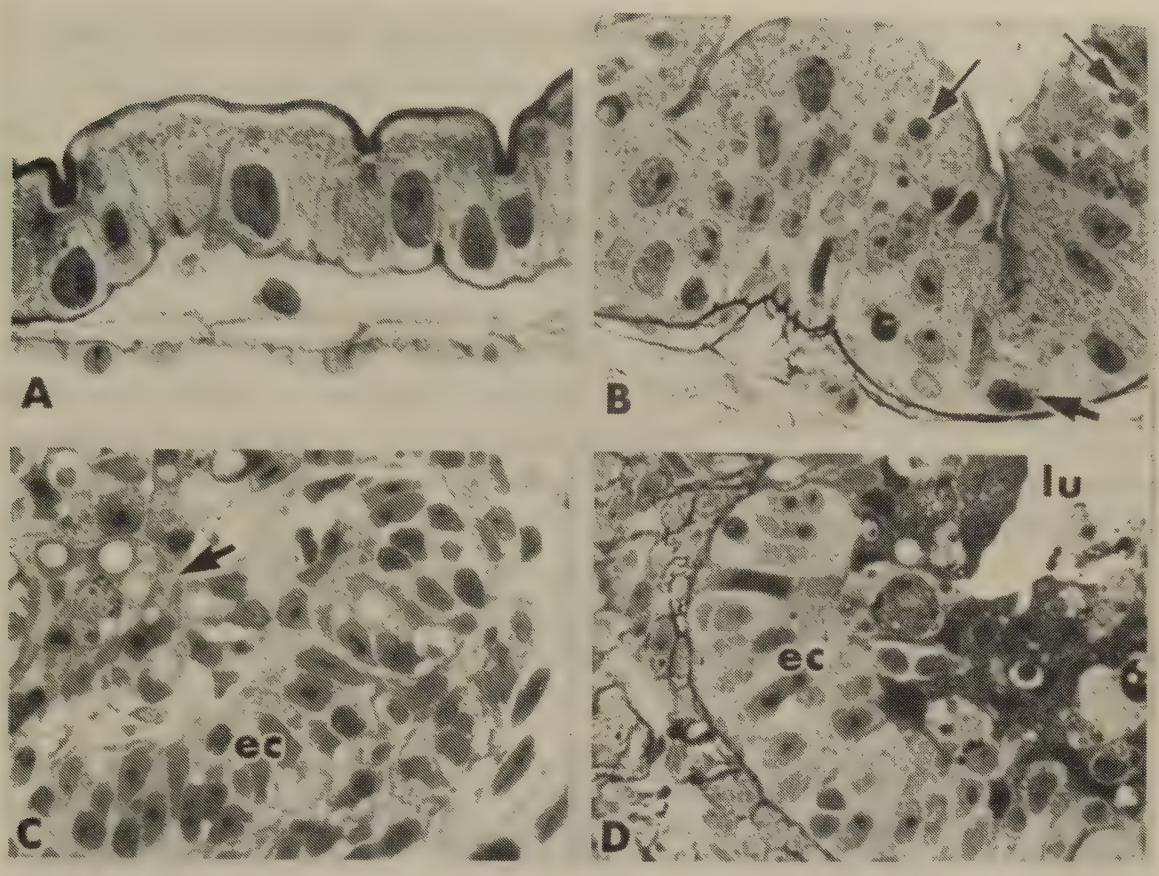



FIG. 24. *Discoglossus* larvae in premetamorphosis treated with $T_4$. Semithin sections. (A) Primary epithelium, control. ×350. (B) Sixteen-hour treatment. Chromophilic lysosomes appear in the primary epithelium (thin arrow). At its base, an embryonic cell is occasionally observed (thick arrow). ×350. (C) Four-day treatment. Islets of embryonic cells (ec) push the primary epithelium (arrow) toward the center of the gut. ×350. (D) Six-day treatment. On the internal border of an islet (ec), the highly chromophilic, lysed primary epithelium is being evacuated into the intestinal lumen (lu). ×350. (From Hourdry, 1971d.)

## A. IMMERSION IN A $T_4$ SOLUTION

*Discoglossus* tadpoles in premetamorphosis were chosen. Some animals were placed in an aqueous $T_4$ solution at $10^{-7}$ gm/ml for periods ranging from 3 hours to 7 days. Control animals were placed in tap water for the same amounts of time. The temperature was 20 ± 1°C.

### 1. *Morphological Modifications*

Treatments lasting less than 1 day do not modify the gut, which remains long, spiral, and of large diameter. Once a period of 24 hours is exceeded, the organ begins a process of reduction in length and diameter, as well as despiralization.

2. *Histological Study of the Intestinal Epithelium*

a. *Light Microscopy.* No reaction of the primary epithelium can be detected following treatment lasting 15 hours or less. Beyond this period, chromophilic lysosomes of variable size can be detected (Fig. 24B). Basal cells multiply actively after a lag of 3 days, generating islets which push the primary epithelium toward the center of the gut (Fig. 24C). These islets will constitute a secondary replacement epithelium, while the degenerating primary epithelium will cast its remnants into the lumen (Fig. 24D). The same intestinal changes in control animals require 2 months at 20°C.

b. *Electron Microscopy.* When the treatment is prolonged beyond a period of 16 hours, significant ultrastructural changes are noted in an increasing number of primary epithelium cells.

Cells that are slightly altered contain small primary autolytic vacuoles and their residual forms (Fig. 25), and the Golgi apparatus is more developed.

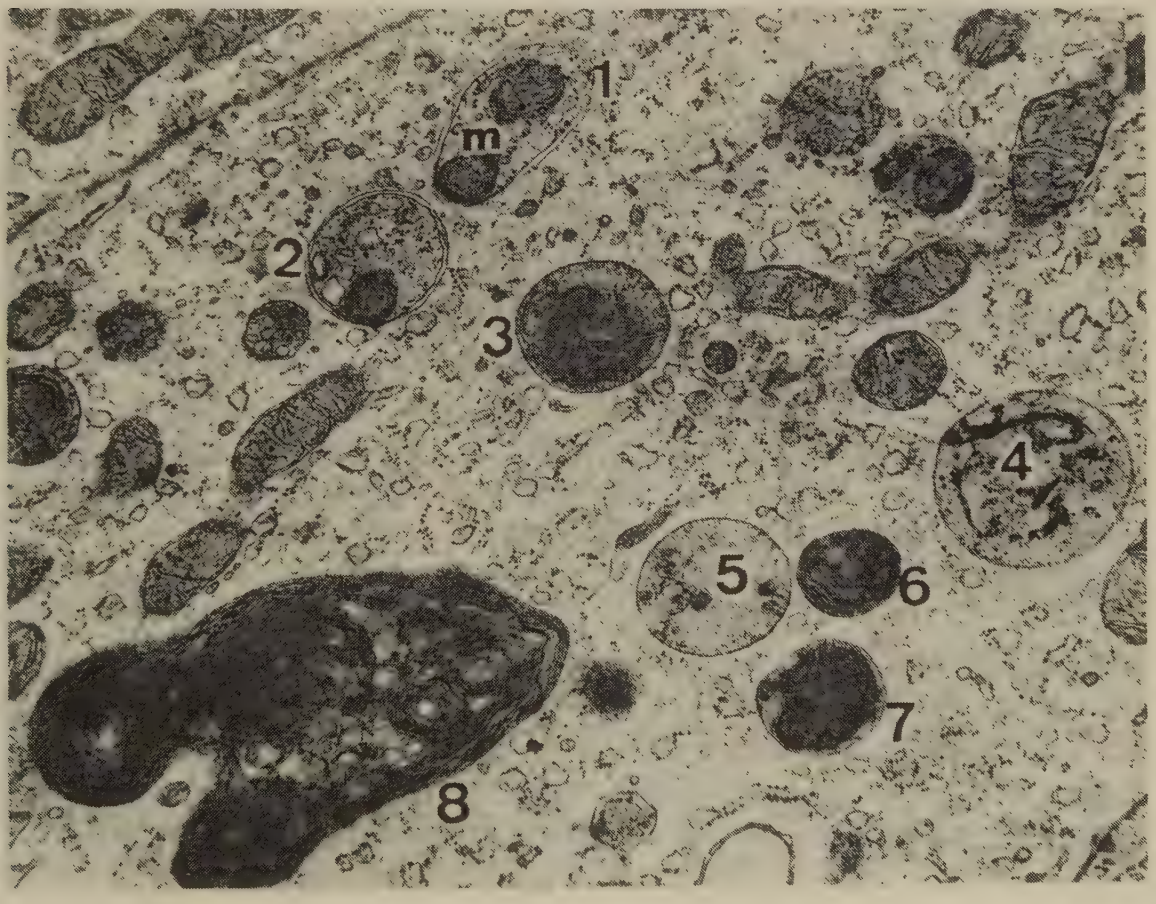



FIG. 25. *Discoglossus* larva in premetamorphosis, treated with $T_4$ for 16 hours; primary epithelium. Primary vacuoles (1–3) contain mitochondria (m). These vacuoles become transformed into primary residual bodies which are either clear (4 and 5) or dense and myeloid (6–8). ×18,500. (From Hourdry, 1971d.)

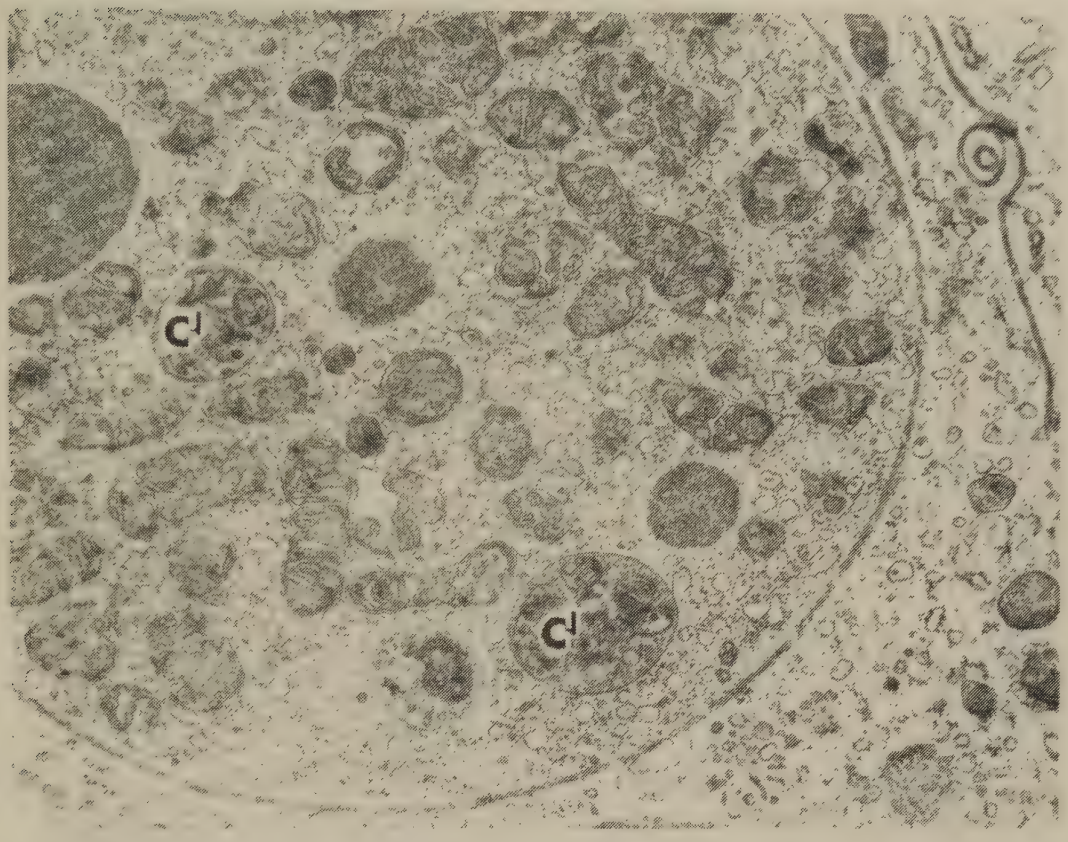



FIG. 26. *Discoglossus* larva in premetamorphosis, treated with $T_4$ for 16 hours; primary epithelium. Primary residual bodies ($C_1$) and mitochondria are segregated in this secondary autolytic vacuole. ×19,000. (From Hourdry, 1971d.)

In more highly altered cells, microvilli become shorter and disappear completely in places. Osmiophilic granules 600–1200 Å in diameter are detected in the enlarged extracellular spaces, vesicles of the reticulum, and Golgi vacuoles. Primary and secondary autolytic vacuoles and residual bodies appear in great numbers (Figs. 26 and 27). These lysosomes have been described during the natural degeneration of the primary epithelium as a part of a defense against the progression of autolysis.

Phagolysosomes are sometimes noted in the interior of still healthy cells and may represent the absorption of neighboring debris.

The most highly altered epithelial cells emit their contents into the intestinal lumen after rupture of the apical cell membrane. The majority of these cells thus disappear after 6 days of treatment, being replaced by secondary epithelium.

### 3. *Localization of Acid Phosphatase Activity*

In controls this activity is localized in fine supranuclear granules in primary epithelium cells (Fig. 28A), increasing in these cells in

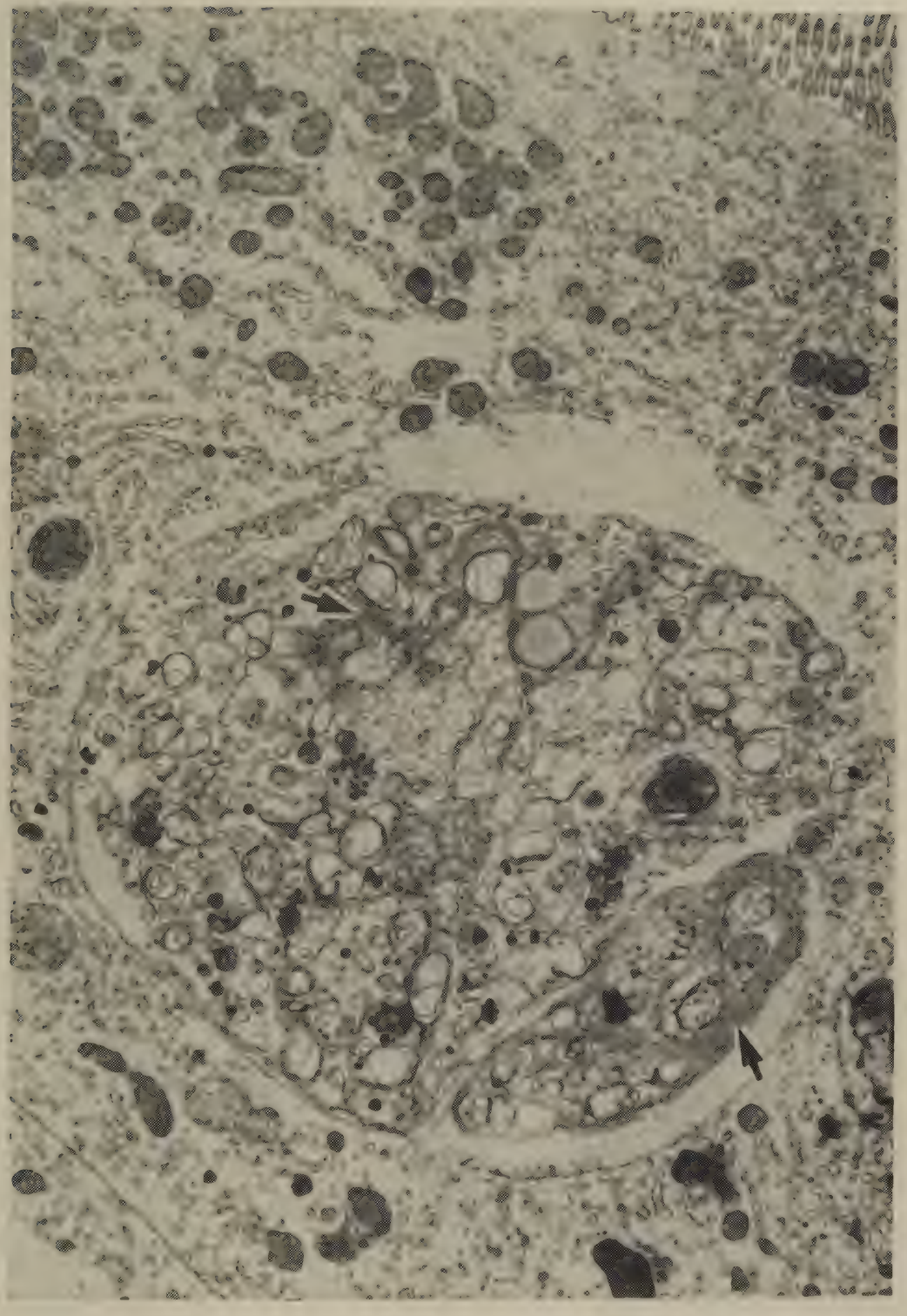


FIG. 27. *Discoglossus* larva in premetamorphosis, treated with $T_4$ for 24 hours; primary epithelium. This secondary residual body, rich in membrane whorls (arrows), has a discontinuous membrane. ×9000. (From Hourdry, 1971d.)

treated animals if the $T_4$ treatment continues beyond 15 hours. Large acid phosphatase-positive lysosomes are seen (Fig. 28B–D and F), which correspond to primary and secondary autolytic vacuoles and their residual forms. These histological data have been confirmed by

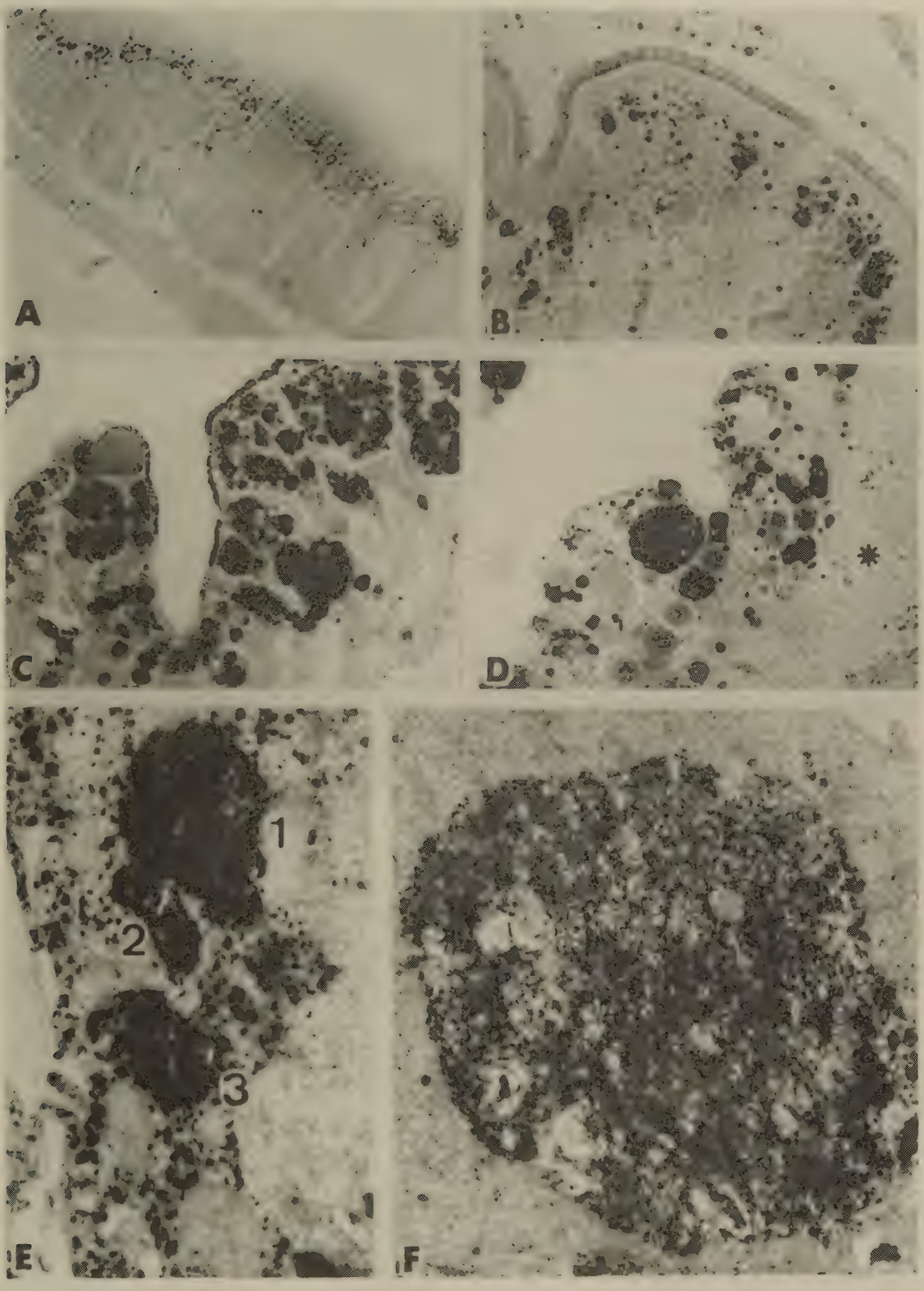
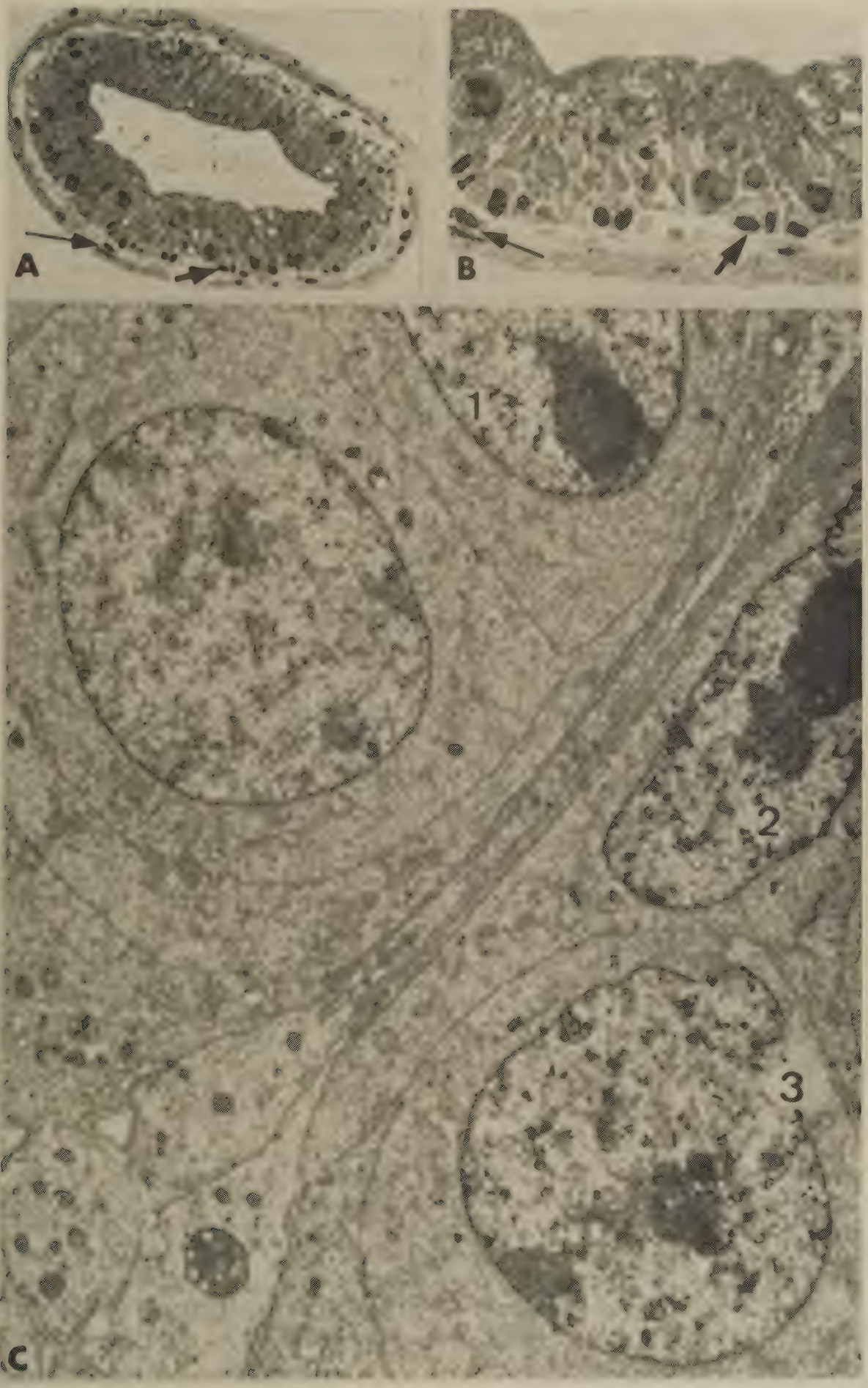



FIG. 28. *Discoglossus* larvae in premetamorphosis, treated with $T_4$. Visualization of acid phosphatase activity in the primary epithelium. (A–D) semithin sections. (A) Control epithelium. ×400. (B) Twenty-four-hour treatment. ×500. (C) Sixty-hour treatment. ×400. (D) Five-day treatment. The secondary epithelium shows no enzyme activity (asterisk). ×400. (E and F) Thin sections. (E) Sixty-hour treatment. The reaction product is located both in lysosomes (1–3) and diffuse in the hyaloplasm. ×15,000. (F) Five-day treatment. Residual body. ×14,000. (From Hourdry, 1971d.)


A
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enzyme assays (Hourdry, 1972c). The activity of the total enzyme peaks if treatment takes place between days 3 and 6. Diffuse activity is also seen in primary epithelium cells undergoing alteration (Fig. 28E). No activity is seen in the secondary epithelium (Fig. 28D).

### 4. *Quantitative Study of the Proliferation of Secondary Epithelium Cells*

During induced metamorphosis of the gut, tritiated thymidine incorporation into the nuclear DNA of a large number of basal islet cells has been demonstrated (Dauça *et al.*, 1976; Fig. 29). The labeling index of the islets reaches a maximum in *Discoglossus* larvae treated for 4 days. *Alytes* larvae exhibit this maximum after 9 days of treatment, and the labeling index approaches 50%. The activity of the DNA has been computed during induced intestinal metamorphosis in *Alytes* (M. Dauça and J. Hourdry, unpublished results); this activity culminates after 7–9 days of treatment.

### 5. *Acquisition of Competence by the Intestinal Epithelium*

When a sufficient concentration of thyroid hormones is administered to anuran tadpoles, they become capable of metamorphosis at a precocious developmental stage (Etkin, 1950; Moser, 1950; Kaye, 1961; Kollros, 1961; Kaltenbach, 1968; Tata, 1968; Knutson and Prahlad, 1971). Virtually complete metamorphosis can be induced as soon as the operculum forms, the thyroid not yet having appreciable activity. Many organs then become competent, that is, capable of responding to the hormone. This capacity is acquired either suddenly (Etkin, 1950) or progressively (Kollros and McMurray, 1956; Heady and Kollros, 1964). In some organs, such as the median eminence of the hypothalamus, however, competence appears later (Etkin, 1966). Both a minimal degree of histological differentiation and the presence of hormonal receptors are necessary (Frieden and Just, 1970; Tata, 1970; Etkin and Gona, 1974). The exact moment of the acquisition of competence by the gut has not been determined, but it appears that it occurs

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FIG. 29. Larvae in premetamorphosis treated with $T_4$; intestinal epithelium following administration of tritiated thymidine. (A and B) Semithin sections. (A) *Discoglossus*. Hormone treatment for 4 days; injection of 6 $\mu$Ci of precursor. Labeled nuclei are often found in the embryonic islets (thick arrow). The nuclei of several connective and muscle cells are also labeled (thin arrow). ×140. (B) Detail of (A). ×350. (C) *Alytes*. Hormone treatment for 11 days; injection of 24 $\mu$Ci of precursor. Silver grains are localized on nuclei 1–3 of the embryonic islets. ×5000.

very early in development; an epithelial response has always been obtained in the youngest individuals examined (stage III in the table of Taylor and Kollros, 1946).

6. *Mechanism of Action of Thyroid Hormones*

a. *Direct Action and Specific Response*. Direct action of thyroid hormones on various amphibian organs has been demonstrated (Kaltenbach, 1968; Kaltenbach and Hobbs, 1972). Several series of experimental results have firmly established this action in the intestine. Local degeneration of intestinal primary epithelium has been induced following the implantation of cholesterol pellets enriched in $T_4$ (Barry, 1952; Lipson, 1965). When labeled hormones are injected into tadpoles, elevated amounts of radioactivity appear in the gut undergoing changes (Ashley and Frieden, 1972; Dratman, 1974). Finally, alterations occur in primary epithelium cells following treatment with $T_4$ *in vitro* (Pouyet *et al.*, 1976); the necrotic figures observed resemble those obtained during spontaneous and induced tadpole metamorphosis.

The results for induced metamorphosis accentuate the specific response to $T_4$ by the two intestinal epithelial cell populations; one degenerates through autolysis, while the other undergoes active multiplication, beginning with the basal stem cells (Bonneville, 1963; Hourdry, 1972a).

b. *Active Form of the Hormones*. The actual form in which thyroid hormones act in the interior of the target cells is still a matter for discussion. The possibility of a preceding deiodination has been suggested but is currently being questioned (Etkin, 1968; Frieden and Just, 1970; Ashley and Frieden, 1972; Griswold *et al.*, 1972; Dratman, 1974; Kistler *et al.*, 1975). No deiodinated derivative of thyroid hormones has been detected in peripheral tissues, whatever the developmental stage of the anuran larvae (Ashley and Frieden, 1972). Although $T_4$ deiodinase activity has been detected in various viscera during metamorphosis (Yamamoto, 1964), this activity is not sufficiently high to result in the liberation of iodine from $T_4$. In a more general fashion, these hormones undergo no chemical transformations (Griswold *et al.*, 1972; Yoshizato *et al.*, 1975a). A role for the iodine in thyroid hormones is suggested as a result of these considerations; this role is, according to Frieden and Yoshizato (1974), limited to the synthesis of $T_3$ and $T_4$. Contrary to what was thought several years ago, the function of these hormones does not seem to depend on the presence of iodine.

c. *Sites of Action*. In the intestinal epithelial cells of anuran larvae these sites are not presently known. Nevertheless, interesting results have been obtained at the level of the liver, tail, and neuroaxis, where

binding with cytoplasmic macromolecules (probably acidic proteins) has been demonstrated between $T_3$ and $T_4$ (Dratman *et al.*, 1969; Griswold *et al.*, 1972; Yoshizato *et al.*, 1975b). Such sites, however, have a low affinity for the hormones. $T_3$ and $T_4$ could become concentrated at the level of intracellular membranes, in association with proteins or, perhaps directly, with phospholipids (Hillier, 1970). The hormones are also fixed at nuclear sites which have a high affinity but a low capacity (Griswold *et al.*, 1972; Kistler *et al.*, 1975; Yoshizato *et al.*, 1975a). In the nuclei of caudal cells, the maximum binding capacity for $T_3$ increases twofold during metamorphosis, thus conferring on these cells a greater reactivity toward the hormone (Yoshizato and Frieden, 1975).

d. *Hormone Action on Genetic Mechanisms.* This action is quite well established in the tail and liver. The binding of thyroid hormones at nuclear sites in liver cells leads to the elaboration of new genetic information with attendant stimulation of transcription and translation (Frieden and Just, 1970; Cohen, 1970; Atkinson, 1971; Griswold *et al.*, 1972; Tata, 1974). Early activation of the synthesis of nuclear RNAs and, primarily, rRNA has been demonstrated in hepatocytes. The concentration of cytoplasmic RNAs subsequently increases. The synthesis of phospholipids and structural proteins accelerates, leading to a proliferation of rough endoplasmic reticulum profiles (Bennett *et al.*, 1970) and the appearance of new proteins, including enzymes of the urea cycle.

The mechanism of thyroid hormone action during tail involution has been investigated with the use of protein synthesis inhibitors such as actinomycin D (Eeckhout, 1969; Weber, 1969; Frieden and Just, 1970; Tata, 1974). By preventing RNA synthesis, this drug suppresses the production of hydrolases which are responsible for the destruction of caudal tissues.

Our knowledge of comparable processes in the gut is still fragmentary. In order to obtain an increase in the number of lysosomes and in acid phosphatase activity in the primary epithelium, a $T_4$ treatment of at least 16 hours is necessary (Hourdry, 1971d). This lag is consistent with hormonal action on transcriptional and translational processes. More direct arguments confirm this action. The beginning of rough endoplasmic reticulum organization *in vitro* is observed near the nucleus of primary epithelium cells when the explants are treated with $10^{-8}$ gm/ml of $T_4$; polysomes are also present (Pouyet *et al.*, 1976). These observations are consistent with increased synthesis of lysosomal hydrolases, already suggested by histochemical results. During $T_4$-induced metamorphosis, primary epithelium cells degenerate, while secondary epithelium cells multiply (Hourdry, 1971d).

This specific response is another argument in favor of hormone action at the level of genetic mechanisms.

e. *Hormone Action at the Cytoplasmic Level*. These hormones may act on the structure and permeability of the lysosomal membrane (Gahan, 1967), thus permitting the diffusion of hydrolases into the hyaloplasm of primary epithelium cells. This hypothesis has been supported by the observations of Hillier (1970), which showed that $T_3$ and $T_4$ could be bound to lipoprotein membranes.

Perturbations of mitochondrial phosphorylation by $T_4$ have been reported (Gustafsson *et al.*, 1965; Gruenstein and Wynn, 1970). The role of respiratory variations in the induction of degenerative processes should not, however, be overestimated. A modification in energy metabolism seems to be excluded as an initial event in anuran metamorphosis (Weber, 1969).

## B. Thyroidectomy and Hypophysectomy

The hypophysis acts on the thyroid gland through secretion of thyrotropic hormone, thus governing the changes of metamorphosis (Etkin, 1968). Cauterization or ablation of the hypophysis blocks all transformations and maintains thyroid activity at a low level.

Levels of circulating thyroid hormones have been artificially modified by performing thyroidectomies or hypophysectomies on *Xenopus* larvae (Hourdry, 1972b). The relative transparency of these animals allows such operations to be performed under more favorable conditions than in other anuran larvae. Animals at the beginning stage of prometamorphosis were either operated on using the technique of Regard and Mauchamp (1971) or were retained as nonoperated controls. Operated tadpoles were placed in tap water at 20 ± 1°C and sacrificed 8–16 days later, at the same time as the controls.

### 1. *Effects of Excisions on the Intestinal Epithelium*

Thyroidectomized or hypophysectomized tadpoles undergo no transformations from the moment of the operation onward (Hourdry, 1972b). No important sign indicative of alterations is perceived in the intestinal primary epithelium cells (Fig. 30A and C). Small lysosomes, not exceeding 2 $\mu$m in diameter, are located in the supranuclear space (Fig. 31). Occasionally, the beginning of rough endoplasmic reticulum organization is seen (Fig. 31). Control animals, on the contrary, reach a climax, their primary epithelium degenerating and being replaced by secondary epithelium (Fig. 30B and D).

The intestinal primary epithelium cells of operated larvae show weak acid phosphatase activity, visible in the apical lysosomes. Con-

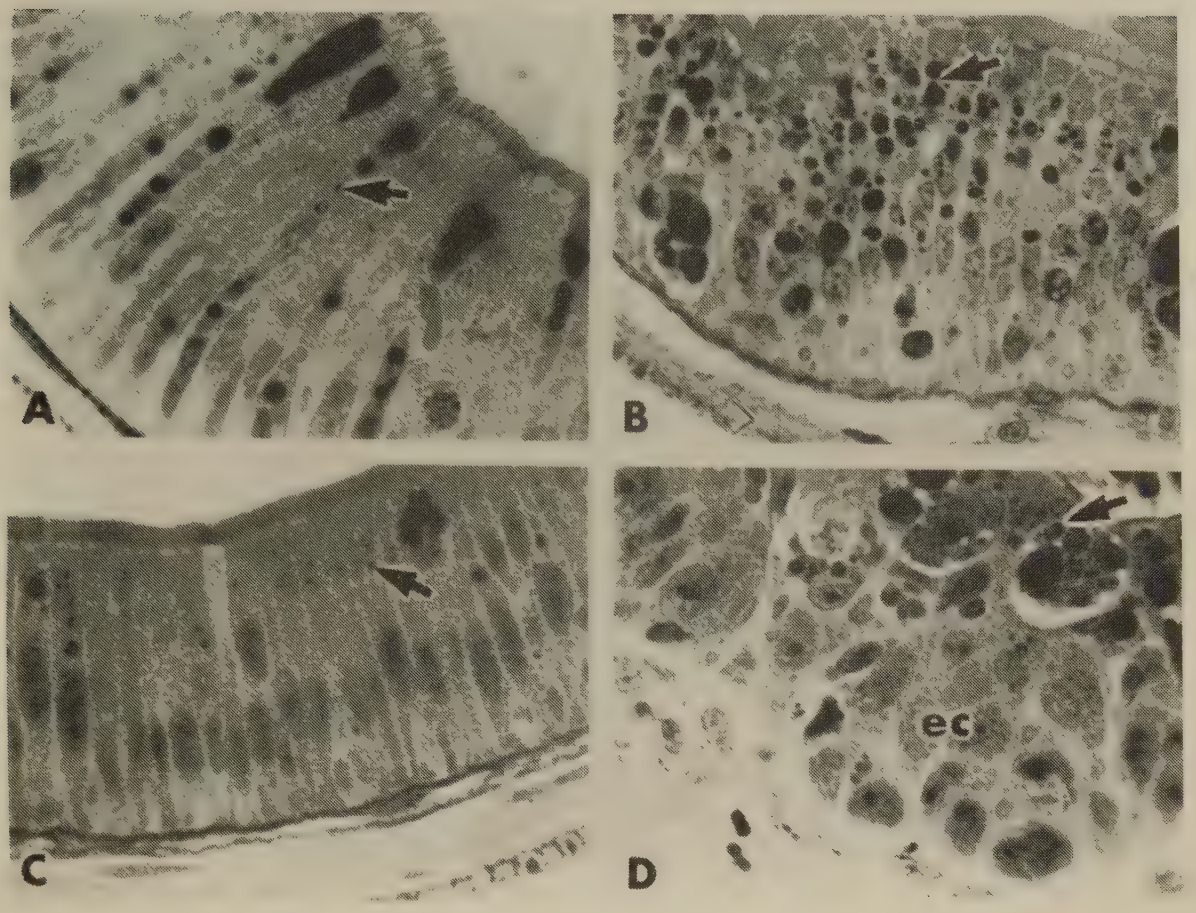


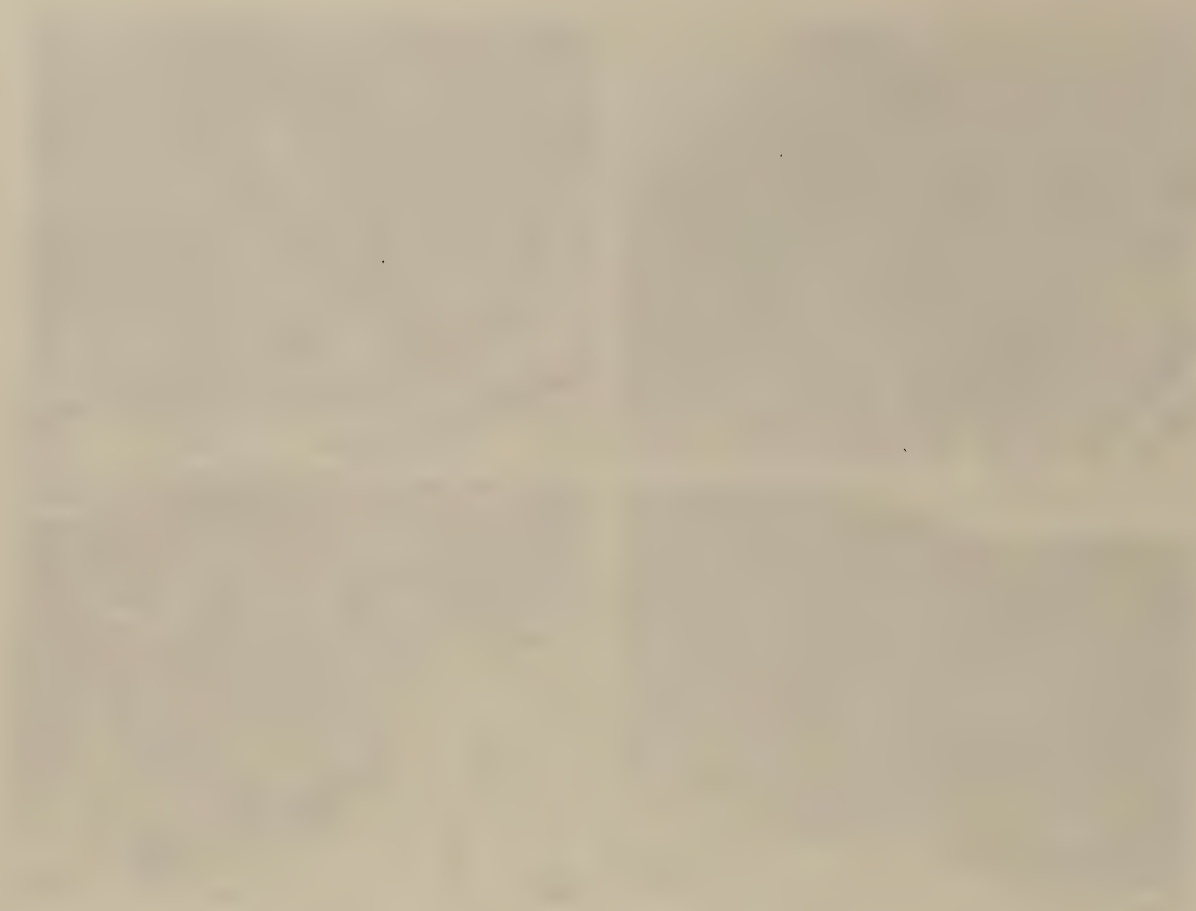

FIG. 30. Semithin sections of the intestinal wall in *Xenopus* larvae. (A) Thyroidectomy at stage 57. After 10 days, the supranuclear space of the primary epithelium cells contains only several lysosomes (arrow). ×550. (B) Primary epithelium of a nonoperated control. Lysosomes are large and frequent (arrow). ×400. (C) Hypophysectomy at stage 57. Eight days after the operation, lysosomes are small and rare in the primary epithelium cells (arrow). ×500. (D) Primary epithelium of a nonhypophysectomized control. Embryonic cells (ec) multiply actively beneath the necrotizing primary epithelium (arrow). ×400. (From Hourdry, 1972b.)

trol epithelium is endowed with much more intense activity, seen both in large lysosomes and diffusing in the hyaloplasm.

It should be added that the administration of thyrotropic hormone to hypophysectomized larvae leads to epithelial transformations (Hourdry, 1973c).

### 2. *Pattern of Thyroid Activity during Thyroidectomy*

During prometamorphosis, thyroid volume increases (Etkin and Lehrer, 1960; Etkin, 1968), follicular epithelium thickens (Van Oordt, 1966; Regard and Mauchamp, 1971), the ergastoplasm becomes organized into superimposed and infranuclear cisternae (Regard and



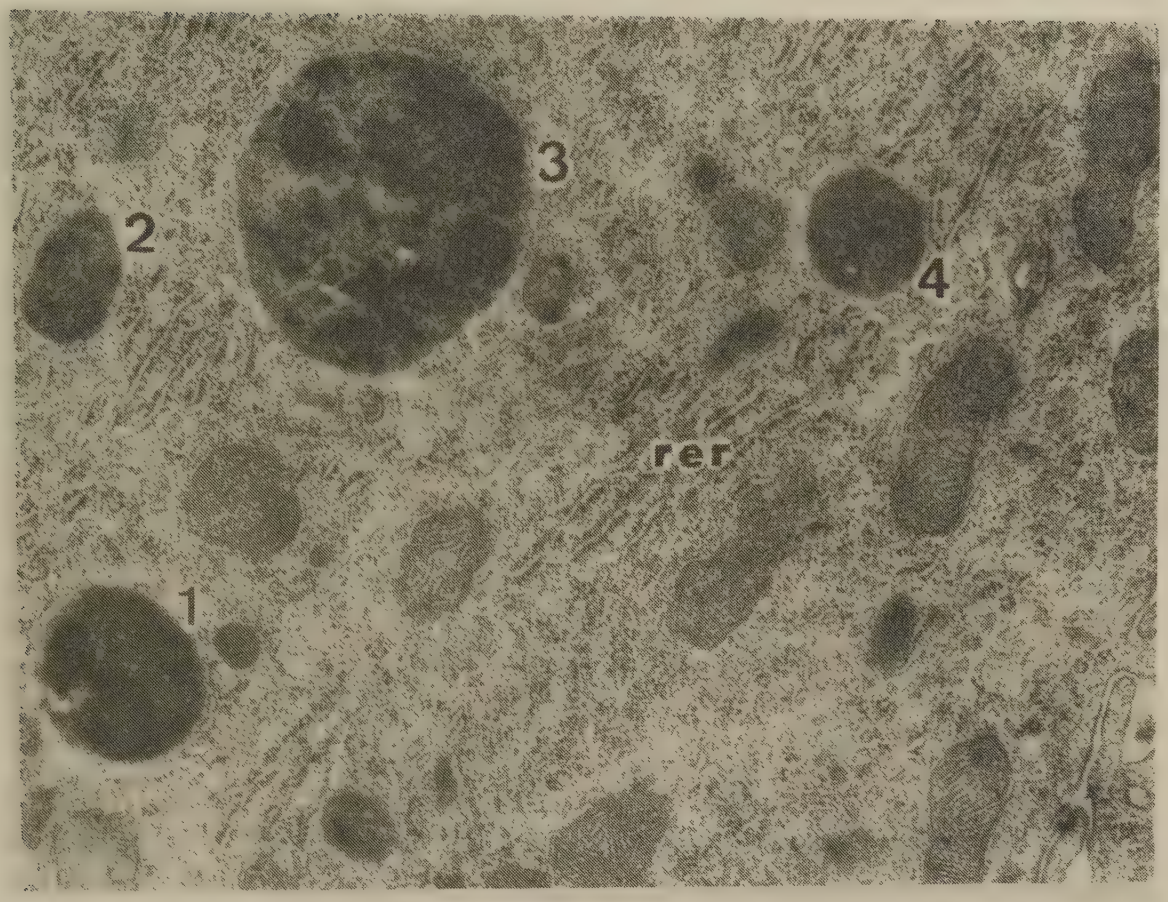



FIG. 31. *Xenopus* larva at stage 57 thyroidectomized 10 days before; primary epithelium. Several dense bodies (1–4) are seen in the supranuclear space. The beginning of rough endoplasmic reticulum organization (rer) is also seen. ×22,500. (From Hourdry, 1972b.)

Mauchamp, 1971), the incorporation of iodine accelerates (Kaye, 1961), and the level of newly synthesized thyroglobulin increases (Regard and Mauchamp, 1971). Thyroidectomy was thus performed during increasing thyroid activity. The drop in hormone concentration was perceived indirectly, through the arrest of intestinal epithelium cell evolution.

### 3. *Influence of Hypophysectomy on Thyroid Activity during Prometamorphosis*

Various workers have shown that hypophysectomy performed during prometamorphosis leads to a decrease in thyroid activity. Turner (1969) reported a delay in the growth of the gland. In addition, Regard and Mauchamp (1971) detected differences in operated larvae with respect to controls: The follicular epithelium flattened, there was an almost total regression of the ergastoplasm, and thyroglobulin synthe-

sis dropped significantly. Although not verified assay, the reduction in this activity suggests a decrease in the level of circulating thyroid hormones. As in the case of thyroidectomy, indirect proof is furnished by stasis of the intestinal epithelium.

### C. Treatment with an Antithyroid Agent, the Perchlorate Ion

Various antithyroid agents (thiourea, thiouracil, perchlorate ion, and so on) have been utilized to slow down or inhibit metamorphosis (Gordon *et al.*, 1945; Pflugfelder and Schubert, 1965; Coleman *et al.*, 1967; Fox and Turner, 1967; Goos, 1969; MacGarry and Vanable, 1969; Mira-Moser, 1969). The perchlorate ion has been used to study this inhibition in the intestinal epithelium (Hourdry, 1973b). This ion prevents the thyroid from concentrating iodide (Greer *et al.*, 1966), perhaps by a competitive inhibition mechanism (Wolff, 1964), which leads to a drop in the level of circulating hormones (Coleman *et al.*, 1967; MacGarry and Vanable, 1969).

Tadpoles of *Discoglossus* were chosen that were either in premetamorphosis or at the beginning of prometamorphosis. They were placed in a 0.1% aqueous solution of potassium perchlorate for periods ranging from 4 to 35 days. As a consequence of the reduction in thyroid activity, the perchlorate ion decreases the developmental rate of larvae and prevents shortening and despiralization of the gut. The primary epithelium cells of treated larvae show no significant signs of alteration. At the very most, several small autolytic vacuoles and their residual forms can be seen. Acid phosphatase activity remains strictly localized in these lysosomes. In controls kept in tap water at the same temperature (20 ± 1°C), however, the intestinal epithelium is the site of important transformations.

### D. Conclusion

Treated animals belong to two categories. The first includes larvae whose level of circulating thyroid hormones is lowered by thyroidectomy, hypophysectomy, or antithyroid treatment. No appreciable alteration of intestinal primary epithelium is observed. Larvae in the second category are those in which the concentration of plasma $T_4$ is increased during immersion in a solution of this hormone. The intestinal primary epithelium cells of these animals contain various types of lysosomes and undergo degeneration. The basal stem cells multiply and give rise to secondary replacement epithelium.

The intestinal changes seen during spontaneous metamorphosis have features in common with those observed during induced

metamorphosis. They are triggered by an increase in the concentration of circulating thyroid hormones appearing during prometamorphosis. The hormones can in both cases accelerate the synthesis of lysosomal hydrolases in primary epithelium cells by acting on genetic mechanisms. The degeneration of these cells, caused by the diffusion of lytic hydrolases into the hyaloplasm, follows a change in the properties of the lysosomal membrane induced by the hormones. The same hormones trigger inversely the multiplication of secondary epithelial cells.

## IV. General Conclusions

The intestinal epithelium of anuran amphibians undergoes significant changes at the paroxysm of metamorphosis (climax), at the moment when the forelimbs emerge and the tail begins to regress.

The primary epithelium degenerates, its cells containing numerous, sometimes large, lysosomes consisting of autolytic vacuoles and their residual forms. These lysosomes can be interpreted as being part of a defense reaction against the progression of degeneration. The material undergoing lysis is extruded into the intestinal lumen following rupture of the apical cell membrane, and a secondary epithelium replaces the primary epithelium.

As with all metamorphosis-related events, the transformations of the intestinal epithelium are triggered by an increase in the level of plasmic thyroid hormones, occuring during prometamorphosis. If the concentration of these hormones is maintained low after thyroidectomy, hypophysectomy, or treatment with an antithyroid agent, the primary epithelium will show no important signs of alteration. On the contrary, when the level of circulating $T_4$ is artificially increased, the primary epithelium degenerates and another epithelium replaces it.

A spontaneous or artificial increase in the level of these plasmic thyroid hormones leads to an increase in the activity of several lysosomal hydrolases in the primary epithelium cells (acid phosphatase, and so on). These enzymes are initially localized in small lysosomes of accumulation. It is suggested that increased synthesis of these hydrolases occurs following hormone action on genetic mechanisms. In addition, the hormones could alter the lysosomal membrane, thus enabling hydrolases to diffuse into the hyaloplasm. Thyroid hormones, by a different and specific action, induce the multiplication of embryonic stem cells and the appearance of secondary epithelium.

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# ISOLEMENT DE L'EPITHELIUM INTESTINAL CHEZ LE CRAPAUD ACCOUCHEUR A L'ETAT LARVAIRE OU JUVENILE.

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# Isolement de l'épithelium intestinal chez le crapaud accoucheur à l'état larvaire ou juvénile

Michel DAUÇA et Jacques HOURDRY

*Laboratoire de Biologie Vertébrés, Université Paris XI, 91405 Orsay, Cedex, France.*
*Laboratoire de Biologie Animale, Ecole Normale Supérieure, 2, avenue du Palais, 92210 Saint Cloud*

**Key words** : *Intestinal epithelium - Separation - Road*

## INTRODUCTION

Lors de la métamorphose naturelle ou provoquée des larves d'Anoures, de nombreux tissus prolifèrent, se différencient ou dégénèrent en présence des hormones thyroïdiennes (Weber, 1977 ; Hourdry et Dauça, 1978).

La réponse d'un organe constitue en fait la résultante des réponses morphogénétiques ou biochimiques, obtenues pour chaque tissu composant, ce qui ne facilite pas l'analyse de l'action des hormones au niveau cellulaire.

Les hormones thyroïdiennes provoquent plus particulièrement de profonds remaniements intestinaux : l'épithélium initial ou primaire dégénère et est remplacé par une assise secondaire de néoformation ; d'autre part, la zone extra-épithéliale s'épaissit fortement, à la fois par hyperplasie et hypertrophie cellulaires (Dauça et Hourdry, 1977). Ces observations sont compatibles avec la synthèse accélérée de diverses espèces moléculaires dans l'épithélium secondaire et dans la zone extra-épithéliale. Afin de mieux analyser ultérieurement l'action des hormones thyroïdiennes sur les synthèses s'installant dans l'épithélium, dont celles des ARN, nous avons cherché à isoler ce tissu des assises plus périphériques.

## MATÉRIEL ET MÉTHODES

Les opérations ont été réalisées sur des Alytes (*Alytes obstetricans* Laurenti) prélevés dans diverses lavognes de la région du Larzac et conservés au laboratoire à 20° ± 1 °C. Leur stade est apprécié au moyen de la table de Taylor et de Kollros (1946).

L'intestin est traité de la manière suivante :

### *Isolement en présence de collagénase*

La préparation de lames épithéliales isolées est obtenue selon une variante de la technique de Carasso *et coll.* (1971). L'intestin est débarrassé de son contenu et placé de 60 à 90 mn, à 20 °C, dans un milieu de culture (Leibovitz, 1963) additionné de collagénase (43 U/ml, type III de Sigma). La séparation de l'épithélium est obtenue sous la binoculaire, à l'aide de pinces fines et d'un micro-scalpel, après ouverture longitudinale de l'organe. Ce tissu est soumis à une double fixation : glutaraldéhyde-acide osmique en présence de tampon phosphate (Dauça et Hourdry, 1978).

### *Séparation de l'épithélium intestinal par l'EDTA*

L'intestin est traité selon une technique dérivée de celle de Weiser (1973). Il est ouvert longitudinalement et nettoyé dans une solution de Holtfreter dépourvue de $Ca^{2+}$, à 4 °C, puis incubé à la température du laboratoire, pendant 10 mn dans cette solution additionnée d'EDTA (5 mM, Sigma). A la fin de l'incubation, l'organe est replacé dans la solution de Holtfreter froide et disséqué sous la binoculaire. L'ensemble de l'épithélium est prélevé au moyen des pinces fines, après glissement sur les couches extra-épithéliales. L'épithélium est fixé comme précédemment.

Des coupes semi-fines et fines sont confectionnées à l'aide d'un ultramicrotome Reichert OMU 2. Les coupes semi-fines sont colorées par le bleu de toluidine-pyronine. Les coupes fines sont contrastées par l'acétate d'uranyle et le citrate de plomb. Les observations ultrastructurales sont effectuées à l'aide d'un microscope Jeol JEM 100 B.

## RÉSULTATS

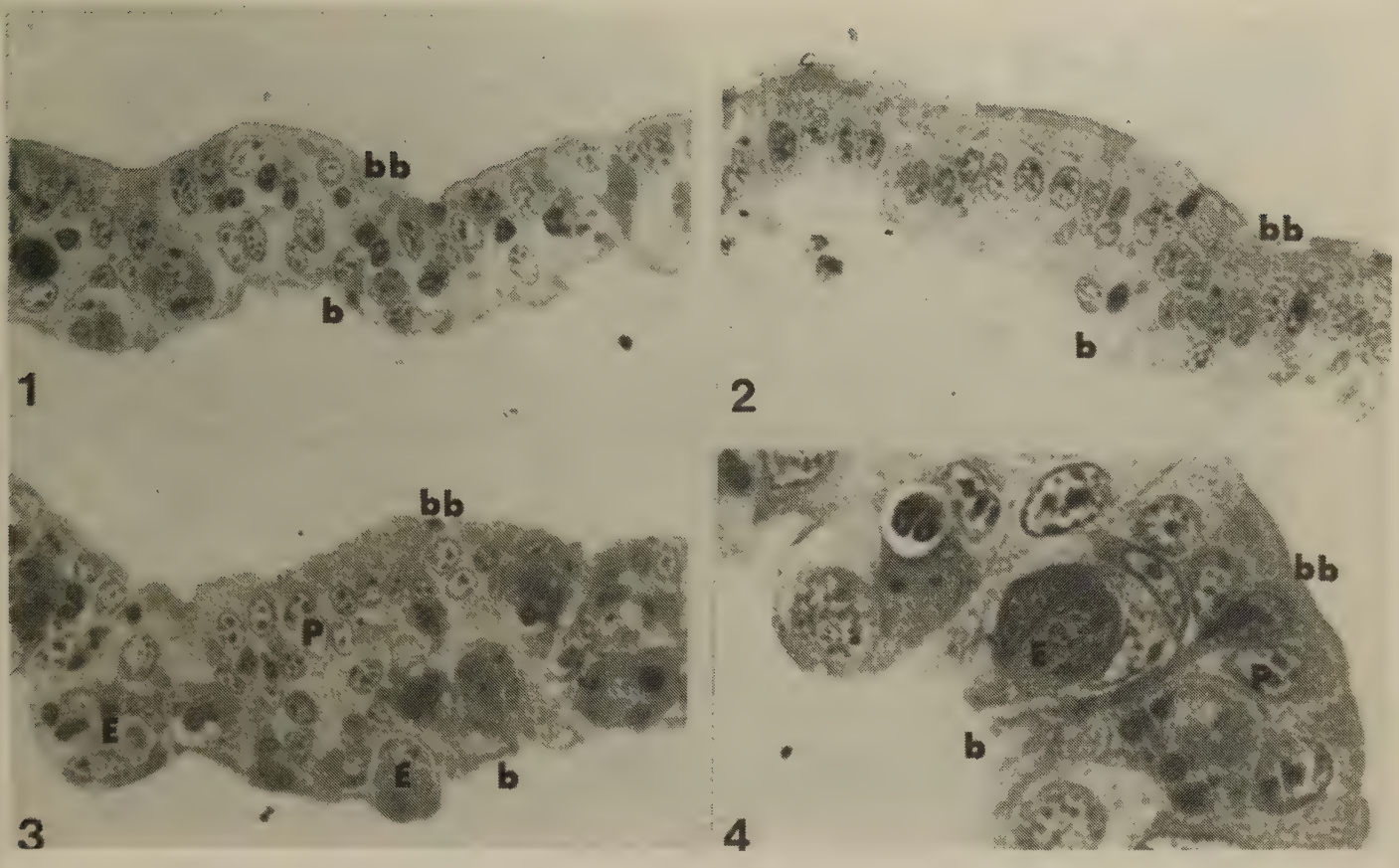
### *Etude morphologique*

Le traitement de l'intestin par la collagénase (Fig. 1, 3, 4) ou l'EDTA (Fig. 2) assure une dissociation de sa paroi et permet d'obtenir une séparation satisfaisante de l'épithélium.

Après un traitement d'une heure par la collagénase, le décollement est souvent imparfait et des lambeaux épithéliaux sont obtenus. 60 mn constituent en fait un temps d'incubation minimal. Par contre, après 90 mn de traitement enzymatique, la dissociation est beaucoup plus prononcée sans être cependant totale.

Une durée d'incubation de 10 mn de l'intestin en présence d'EDTA est suffisante pour permettre l'isolement sans difficulté de la zone épithéliale.

La collagénase et l'EDTA permettent l'isolement de l'épithélium intestinal quel que soit le stade de développement de la larve. D'une façon générale, cette séparation est plus facile lorsque le tissu est homogène, qu'il s'agisse de l'épithélium primaire de la larve en prémétamorphose (Fig. 1 et 2) ou de l'épithélium secondaire villeux et définitif de l'individu juvénile, ayant achevé sa métamorphose. L'isolement de l'épithélium est cependant possible chez des animaux au climax, alors que le tissu comporte à la fois des épithéliocytes primaires en dégénérescence et des cellules embryonnaires basales en cours de prolifération (Fig. 3 et 4).




FIGURES 1 à 4. — Epithélium intestinal isolé, observé sur coupes semi-fines.

FIGURES 1 et 2. — Epithélium primaire de larves en prémétamorphose.
*Fig.* 1. — Séparation par la collagénase; × 550.
*Fig.* 2. — Séparation par l'EDTA; × 550 .

FIGURES 3 et 4. — Climax de la métamorphose. Séparation par la collagénase. Sous l'épithélium primaire (*P*), on remarque plusieurs îlots de cellules embryonnaires basales (*E*). *Fig.* 3, × 550; *Fig.* 4, × 1 000; *b*, base de l'épithélium, *bb*, bordure en brosse.

### *Etude ultrastucturale*

L'épithélium intestinal isolé et l'épithélium *in situ* ne montrent pas de différences fondamentales, lorsqu'ils sont observés au microscope électronique. La structure des tissus ne semble pas affectée par ces techniques de séparation.

Après traitement par la collagénase, les desmosomes et les « tight junctions » sont respectés (Fig. 5 et 6). L'EDTA à la concentration utilisée peut entraîner un élargissement des espaces intercellulaires et une rupture des desmosomes mais, le plus souvent, ces espaces demeurent étroits, tandis que les « tight junctions » et les desmosomes sont préservés (Fig. 8).

Les cellules embryonnaires basales, groupées en îlots, gardent leur aspect habituel (rapport nucléoplasmique élevé, nucléole volumineux, ribosomes abondants, organites cytoplasmiques peu différenciés), et il n'est pas rare d'y rencontrer des figures mitotiques (Fig. 7).

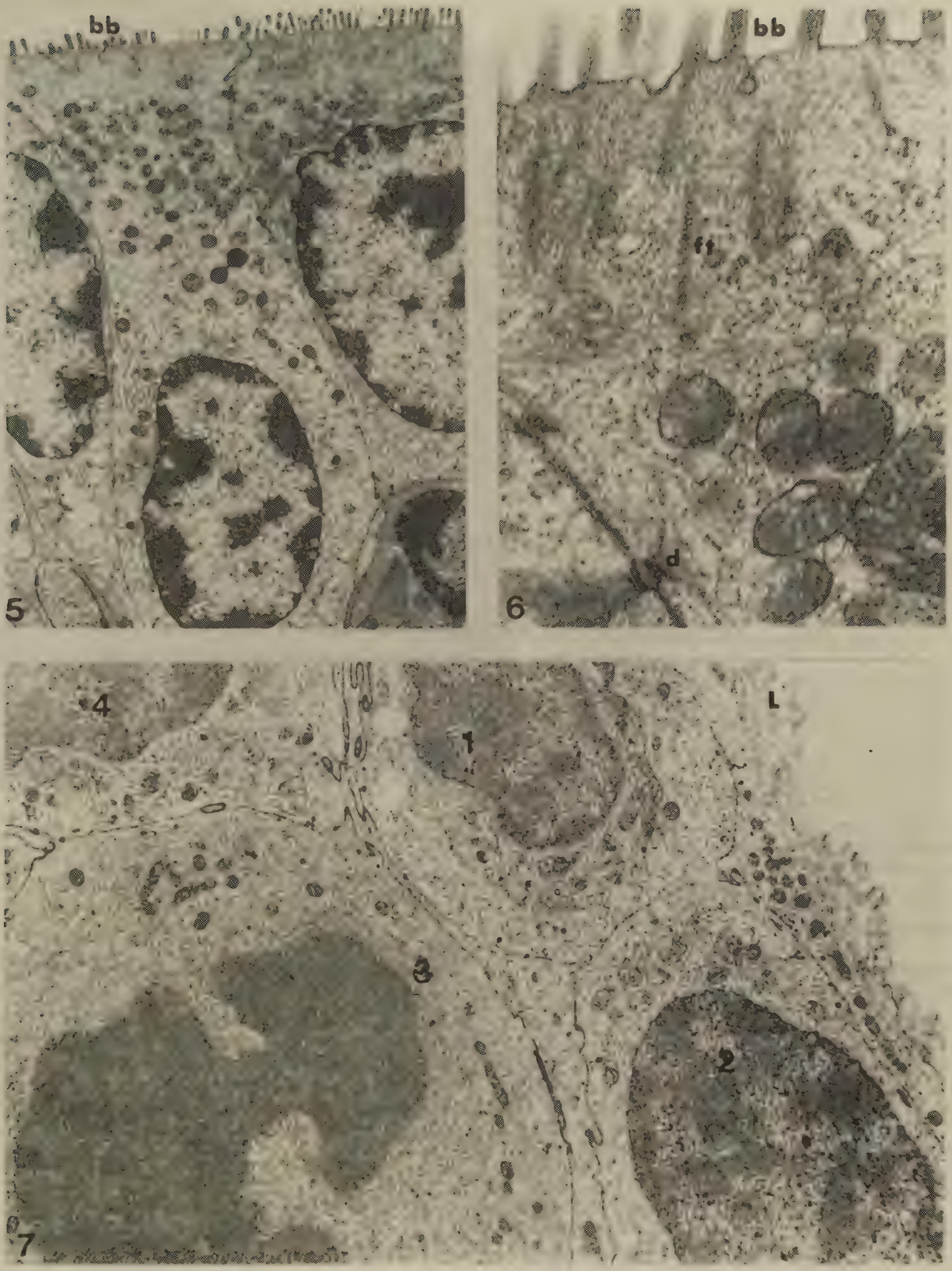
Le plus souvent, la séparation de l'épithélium et de la zone extra-épithéliale est réalisée sur le bord externe de la lame basale. Celle-ci demeure alors associée à l'épithélium et forme de nombreux replis (Fig. 7). Dans quelques cas, toutefois, le clivage s'opère sur le bord interne de la lame basale, et cette dernière reste alors solidaire du chorion. La paroi intestinale présente ainsi deux niveaux de moindre résistance, de part et d'autre de la lame basale.

## DISCUSSION

Chez les Amphibiens larvaires ou adultes, de nombreux auteurs ont réalisé l'isolement de l'épiderme tégumentaire. Certains utilisent une méthode purement physique et l'intervention d'une pression hydrostatique (Rawlins *et coll.*, 1970). D'autres préconisent une méthode strictement chimique avec l'action d'enzymes comme l'élastase (Eisen et Gross, 1965) ou la trypsine (Yvroud et Beaumont, 1974) ou encore l'intervention d'un chélateur comme l'EDTA (Zadunaisky et Lande, 1972). D'autres, enfin, combinent les effets d'une collagénase et d'une pression hydrostatique (Carasso *et coll.*, 1971). Par contre, la séparation de l'épithélium intestinal n'a jamais été effectuée chez les Amphibiens. Ce tissu a seulement été isolé chez le rat, au cours de son développement (Raul *et coll.*, 1977).

• Le présent travail montre qu'il est possible d'obtenir très simplement une séparation efficace de l'épithélium intestinal chez des Anoures en prémétamorphose, en métamorphose ou à l'état juvénile.

• L'intérêt de la technique à l'EDTA réside dans sa rapidité, la faible durée du traitement suggérant une bonne conservation des épithéliocytes. Une méthode voisine, appliquée par Raul *et coll.* (1977) sur l'intestin de rat nouveau-né, a montré que le gradient de concentration de la phosphatase alcaline, de la thymidine kinase et de la sucrase, entre le sommet et la base




FIGURES 5 à 7. — Epithélium intestinal isolé par la collagénase.

FIGURES 5 et 6. — Epithélium primaire de larves en prémétamorphose.

*Fig.* 5. — × 5,900; *Fig.* 6. — × 25,500.

FIGURE 7. — Climax de la métamorphose. Dans cet îlot de cellules embryonnaires basales (1-4), l'une des cellules (3) est en cours de division. La lame basale (*L*) demeure associée à l'épithélium; × 6,500; *bb*, bordure en brosse, *d*, desmosome, *tf*, tonofilament.

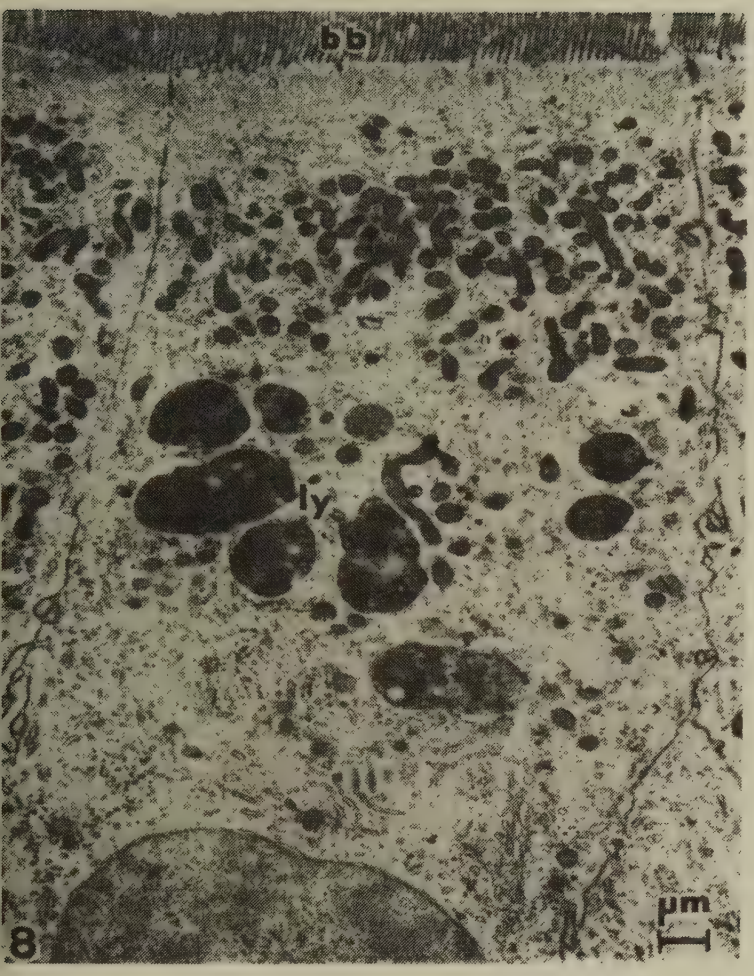



FIGURE 8. — Epithélium intestinal primaire de larve en début de métamorphose, après isolement par l'EDTA (5 mM); × 6 000 *bb*, bordure en brosse, *ly*, lysosome.

des villosités, reste semblable à celui qui existe dans l'intestin *in situ*.

• Une incubation de 60 à 90 mn dans la collagénase conserve les caractères morphologiques des cellules épithéliales. Toutefois, on ne peut affirmer qu'elles préservent toutes les propriétés physiologiques et, en particulier, les capacités de transport des ions, comme cela a été observé au niveau de l'épiderme isolé (Carasso *et coll.*, 1971).

## CONCLUSION

L'isolement de l'épithélium intestinal, chez *Alytes obstetricans* à l'état larvaire ou juvénile, est réalisé en traitant l'intestin par la collagénase ou l'EDTA.

• Le clivage est obtenu de part et d'autre de la lame basale de l'épithélium par une méthode rapide et simple, compatible avec une bonne préservation des épithéliocytes.

• Dans la mesure où chacun des tissus de l'intestin répond aux hormones thyroïdiennes selon des critères spécifiques, l'isolement de l'hépithélium par la collagénase ou l'EDTA est une condition préalable à l'analyse biochimique précise du mode d'action des hormones sur ce tissu-cible, *in vivo* ou *in vitro*.

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ANALYSE QUANTITATIVE DES REMANIEMENTS INTESTINAUX ET EVOLUTION DE LA SYNTHESE DE L'ADN. ETUDE CHEZ LES LARVES D'*ALYTES OBSTETRICANS* ET DE *DISCOGLOSSUS PICTUS* (AMPHIBIENS ANOURES) EN METAMORPHOSE SPONTANEE.

Biologie Cellulaire, 31, n°3, 277-286 (1978).

*Biol. Cellulaire* (1978), **31**, 277-286

# Analyse quantitative des remaniements intestinaux et évolution de la synthèse de l'ADN. Étude chez les larves d'*Alytes obstetricans* et de *Discoglossus pictus* (Amphibiens Anoures) en métamorphose spontanée

Michel DAUÇA et Jacques HOURDRY

*Laboratoire de Biologie Vertébrés (Bâtiment 441), Centre d'Orsay, Université de Paris-Sud, 91405 Orsay, France.*

*Laboratoire de Sciences Naturelles de l'Ecole Normale Supérieure de Saint Cloud, 2, avenue du Palais, 92211 St Cloud, France.*

**During the spontaneous metamorphosis of two anuran Amphibians,** Discoglossus pictus **and** Alytes obstetricans, **rearrangement of the intestinal wall occurs. The relative importance of each wall component is determined on sections. The distribution and frequency of labeled nuclei which synthesize DNA before dividing was examined in larvae after incorporation of tritiated thymidine. The rate of** de novo **DNA synthesis is estimated by measuring its specific activity.**

**During premetamorphosis the initial, or primary, epithelium forms the greatest portion of the wall; the labeling index reaches 8% in this tissue. The specific activity of DNA is high and confirms the rapid growth of the epithelium.**

**At the beginning of metamorphosis (prometamorphosis) the primary epithelium remains preponderant, but it ceases to grow and its nuclei are rarely labeled. The secondary epithelial cells begin to multiply from basal stem cells. Their labeling index is close to 28% but their number is still small. These results are consistent with a highly reduced specific activity of DNA.**

**At the metamorphic climax, the primary epithelium degenerates and disappears. At the beginning of this period, the secondary epithelial cells which arise from the stem cells undergo an active proliferation and one-third of them contain labeled nuclei. The labeling index of the secondary epithelium subsequently decreases and during this period of slower growth, the tissue becomes organized into villi. Cell multiplication is always less intense in the extra-epithelial zone, but nevertheless results in a thickening of this zone, which ultimately occupies half of the wall. Growth processes which appear in the secondary epithelium and in the extra-epithelium zone lead to an increase in the specific activity of DNA.**

**Key-words:** *Anuran - Intestine - Spontaneous metamorphosis.*

## INTRODUCTION

La métamorphose des Amphibiens Anoures est caractérisée par d'importantes transformations morphologiques, histologiques et biochimiques, préparant le passage de l'habitat dulcaquicole à un mode de vie terrestre. (Frieden et Just, 1970). La paroi intestinale est plus particulièrement remaniée. L'épithélium initial dégénère (Bonneville, 1963 ; Hourdry, 1969*a* ; Kopec, 1970), et et cette lyse est provoquée par une augmentation de l'activité de plusieurs hydrolases lysosomales (Brown et Millington, 1969 ; Hourdry, 1971*a* et *b*, 1974 ; Varute et More, 1971 ; Varute Sawant, 1971). L'assise est remplacée par un épithélium de néoformation (Bonneville, 1963 ; Hassan et Marinucci, 1966 ; Hourdry, 1969*b* ; Dournon et Chibon, 1974).

L'analyse quantitative des remaniements intestinaux a fait l'objet du présent travail. Une première approche est réalisée sur coupes semi-fines, en mesurant la surface de chacun des composants de la paroi et en pratiquant des numérations nucléaires. Une seconde investigation est effectuée après incorporation de thymidine tritiée. Elle comporte :

— une analyse radioautographique de la fréquence et de la répartition des noyaux marqués, synthétisant de l'ADN avant de se diviser ;

— le dosage de l'ADN apparu, par recherche de son activité spécifique.

## MATÉRIEL ET MÉTHODES

Les larves de Discoglosse et d'Alyte sont choisies au cours de leur développement naturel, et leur stade est apprécié au moyen de la table de Taylor et Kollros (1946). Durant la prémétamorphose (stades I-XI), les animaux s'accroissent sans subir de changement morphologique notable. Au cours de la prométamorphose (stades XII-XIX), les membres postérieurs s'allongent. Au climax enfin (stades XX-XXV), les événements de la métamorphose s'accélèrent (sortie des membres antérieurs, régression de la queue...), et les larves deviennent des individus juvéniles quittant le milieu dulcaquicole pour un habitat terrestre.

### I. *Techniques histologiques*

Les fragments d'intestin pris à divers niveaux de l'organe sont soit fixés simplement par le tétroxyde d'osmium (Hourdry, 1970) ou subissent une double fixation : glutaraldéhyde-tétroxyde d'osmium en présence de tampon cacodylate (Hourdry, 1971*a*). D'autres fragments sont soumis pendant 25 minutes à 4 °C à une première fixation par le glutaraldéhyde à 2 % dans le tampon phosphate de Sörensen (0,15 M), le pH de ce mélange étant ajusté à 7,4. Après un lavage rapide dans le tampon, les pièces sont post-fixées une heure, à froid, dans un mélange volume à volume de tétroxyde d'osmium (2 %) et de tampon phosphate.

Après déshydratation par l'éthanol, les fragments d'intestin sont orientés dans le milieu d'inclusion (épon 812) afin d'être coupés transversalement.

Les coupes semi-fines de 2,5 µ d'épaisseur sont colorées par le mélange bleu de toluidine-pyronine (Ito et Winchester, 1963). Les coupes fines sont contrastées par l'acétate d'uranyle et le citrate de plomb (Reynolds, 1963).

### II. *Mesure des surfaces et étude des fréquences nucléaires*

Ces mesures sont réalisées sur les coupes semi-fines de 2,5 µ d'épaisseur. Sur ces sections transversales, la paroi de l'intestin peut se décomposer en trois zones :

— l'épithélium primaire ou épithélium de la larve ;

— l'épithélium secondaire. Il se développe dès la prométamorphose et constituera l'épithélium intestinal de l'adulte ;

— la zone extra-épithéliale formée du chorion, de la musculeuse et de l'adventice.

Les contours de chaque zone sont dessinés au microscope optique à projection (Visopan). L'aire représentée par l'ensemble des cellules appartenant à chacune des différentes zones précédemment décrites a été mesurée à l'aide d'un planimètre (Maho). La numération des cellules appartenant aux épithéliums primaire et secondaire est réalisée par comptage direct des noyaux au microscope optique, à partir des mêmes coupes transversales.

### III. *Techniques radioautographiques*

Deux heures avant leur sacrifice, certaines larves reçoivent de la thymidine tritiée par injection intrapéritonéale (6 µCi : Discoglosse ; 24 µCi : Alyte ; activité spécifique : 15 à 26 Ci/mM). Les fragments d'intestin subissent une double fixation. Les radioautographies sont obtenues en microscopie photonique et électronique, par application de la technique de Larra et Droz (1970).

#### A. Microscopie photonique

Les lames sont plongées à l'obscurité dans l'émulsion (Ilford $L_4$) diluée trois fois, séchées et placées dans des boîtes hermétiques de Clay-Adams, à 4 °C, pendant 15 à 18 jours, en présence d'actigel.

La révélation est réalisée par l'amidol (Fluka) chez le Discoglosse et le D. 19 (Kodak) chez l'Alyte. Les coupes semi-fines sont ensuite lavées, fixées dans une solution de thiosulfate de sodium à 20 %, rincées à nouveau et colorées par le mélange bleu de toluidine-pyronine.

#### B. Microscopie électronique

Les coupes fines sont déposées à l'aide d'un anneau sur des lames de verre préalablement recouvertes d'une membrane de celloïdine. Ces coupes sont contrastées par l'acétate d'uranyle (45 minutes) et le citrate de plomb (12 minutes). Après "carbonage", les lames sont plongées dans l'émulsion (Ilford $L_4$) diluée cinq fois, séchées et conservées pendant deux mois dans les mêmes conditions que précédemment. Les radioautographies sont révélées en 4 minutes par le microdol X (Kodak) maintenu à 17 °C puis fixées par une solution de thiosulfate de sodium à 30 %. Une grille est déposée sur chaque ruban de coupes, après décollement du film de celloïdine. Les grilles sont ensuite immergées dans l'acétate d'isoamyle, pendant 3 minutes, afin d'amincir la membrane et d'obtenir une meilleure résolution.

### IV. *Recherche de l'activité spécifique de l'ADN*

Les seules larves d'Alyte sont utilisées car, contrairement à ce que nous observons chez le Discoglosse, la taille de l'intestin est en général suffisante chez l'Alyte pour permettre un dosage par individu. Lors du climax toutefois, les organes sont groupés par deux ou trois, dans cinq cas sur dix, vu leur petite taille. Tous les animaux ont reçu 24 µCi de thymidine tritiée deux heures avant leur sacrifice.

#### A. Extraction de l'ADN total

Elle est réalisée selon la méthode de Munro et Fleck (1966).

L'intestin est vidé de son contenu et broyé dans un homogénéiseur Potter (Bloch), en présence de citrate de sodium (3,2 %) et à la température de la glace fondante. La fraction acido-soluble (acides aminés...) est éliminée par l'acide trichloracétique à 10 %. Les lipides sont extraits successivement par l'éthanol, le mélange chloroforme-éthanol (1-3) et l'éther éthylique. Les ARN sont hydrolysés par la soude (0,3 N) à 37 °C, pendant une heure. En présence d'acide trichloracétique froid à 20 °C, les ribonucléotides restent en solution, tandis que l'ADN et les protéines précipitent. Après une centrifugation à 6 000 t/mn, à 4 °C, pendant 15 minutes, l'ADN du culot est séparé des protéines par mise en solution dans l'acide perchlorique à 0,7 N.

#### B. Dosage de l'ADN total

La densité optique de la solution d'ADN est appréciée avec un spectrophotomètre à UV (Beckman), à la longueur d'onde de 260 nm. La concentration de l'ADN, en µg/ml, est calculée en se référant à une gamme d'étalonnage.

#### C. Activité spécifique de l'ADN

La radioactivité contenue dans 0,2 ml de solution d'ADN est mesurée au moyen d'un compteur à scintillation (Beckman). L'activité spécifique est calculée, compte tenu de la quantité d'ADN présente dans la fraction aliquote, et exprimée en cpm/µg d'ADN. Elle reflète la vitesse d'incorporation du précurseur marqué dans l'ADN.

## RÉSULTATS

### I. *Surfaces des différentes zones de la paroi intestinale*

Les observations sont effectuées chez 29 larves d'Alyte et 17 larves de Discoglosse. Une vingtaine de coupes transversales semi-fines, prises à divers niveaux de l'intestin, sont utilisées par animal. Pour chacune des coupes, la surface des épithéliums primaire et secondaire et de la zone extraépithéliale est exprimée par un pourcentage de la surface de la paroi. Les résultats moyens par période varient de la même façon pour l'un et l'autre espèce. Nous les exposerons plus particulièrement chez l'Alyte (Fig. 1*).

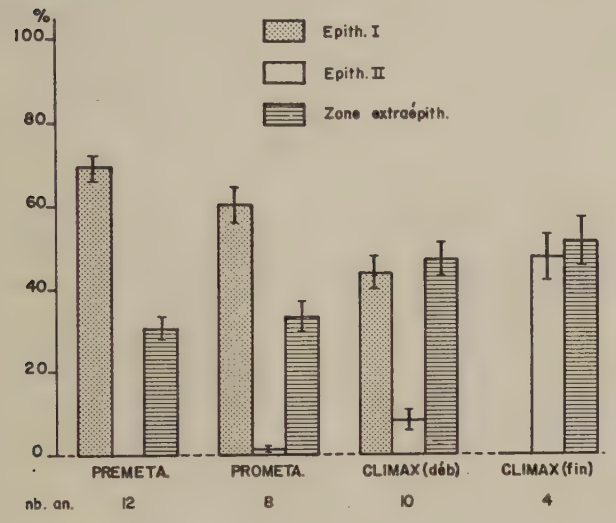


FIGURE 1. — Variations subies par les surfaces de l'épithélium et de la zone extra-épithéliale de l'intestin, chez la larve d'Alyte, au cours de la régression de l'organe (en pourcentages moyens ± erreurs-standard).

Durant la prémétamorphose, l'intestin est long et spiralé. Il présente une large section et une lumière importante. L'essentiel de la paroi est formé par un épithélium primaire sans villosité dont les noyaux sont reconnaissables à leur forme allongée et à leur disposition perpendiculaire à la membrane basale (70 %). Les cellules souches de l'épithélium secondaire sont rares, intercalées à la base des épithéliocytes primaires, peu visibles, ce qui rend difficile la mesure de leur surface. Pendant toute la prémétamorphose et une grande partie de la prométamorphose, ces cellules reposent dispersées contre la membrane basale, ne communiquant jamais avec la bordure en brosse, se reconnaissant par leur forme arrondie et surtout leurs caractères embryonnaires reflétés par leur rapport nucléolasmique élevé, un ou plusieurs nucléoles volumineux, ainsi que la basophilie (toluidinophilie) de leur frange cytoplasmique (Fig. 3*a*). La surface totale de l'ensemble des épithéliocytes secondaires dispersés pendant la prémétamorphose est négligeable. Le chorion et la musculeuse de la zone extra-épithéliale sont très minces, de même que l'adventice.

Au cours de la prométamorphose, aucun changement significatif n'est observé et l'épithélium primaire demeure prépondérant (65 %).

Les remaniements intestinaux importants s'installent au début du climax, au moment où l'animal cesse de se nourrir. L'intestin devient court, subrectiligne et de diamètre plus faible. La surface de la paroi s'accroît toutefois, car la lumière a pratiquement disparu. Le pourcentage représenté par l'épithélium primaire diminue (44 %). Corrélativement, l'épithélium secondaire se développe, mais moins vite chez l'Alyte (9 %) que chez le Discoglosse. Il est alors composé de petits îlots non jointifs, désormais nettement individualisés de l'épithélium primaire (Fig. 3*b*). La zone extra-épithéliale s'épaissit fortement, au point d'atteindre la moitié environ de la surface totale.

A la fin du climax, l'épithélium primaire a totalement régressé. L'épithélium secondaire est maintenant continu, très villeux et constitue presque 50 % de la paroi (Fig. 3*c*).

### II. *Evolution de la population cellulaire dans les deux épithéliums*

Chez les larves d'Alyte, les sections semi-fines utilisées pour la planimétrie ont également servi à calculer les nombres des épithéliocytes primaires et secondaires, que nous avons exprimés par des pourcentages moyens par période (Fig. 2). Le comptage des noyaux est réa-

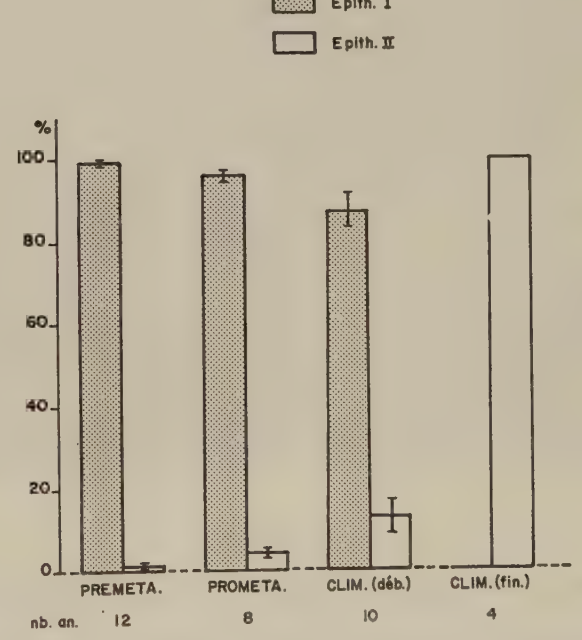


FIGURE 2. — Fréquences nucléaires des épithéliums primaire et secondaire, avant et pendant la métamorphose de la larve d'Alyte (en pourcentages moyens ± erreurs-standard).

Abréviations utilisées dans les Fig. 1, 2, 4 et 7 : CLIM. (déb.), climax (début) ; Epith I, épithélium primaire ; Epith. II, épithélium secondaire ; nb. an., nombre d'animaux ; PREMETA, prémétamorphose ; PROMETA, prométamorphose ; Zone extra-épith., zone extra-épithéliale.

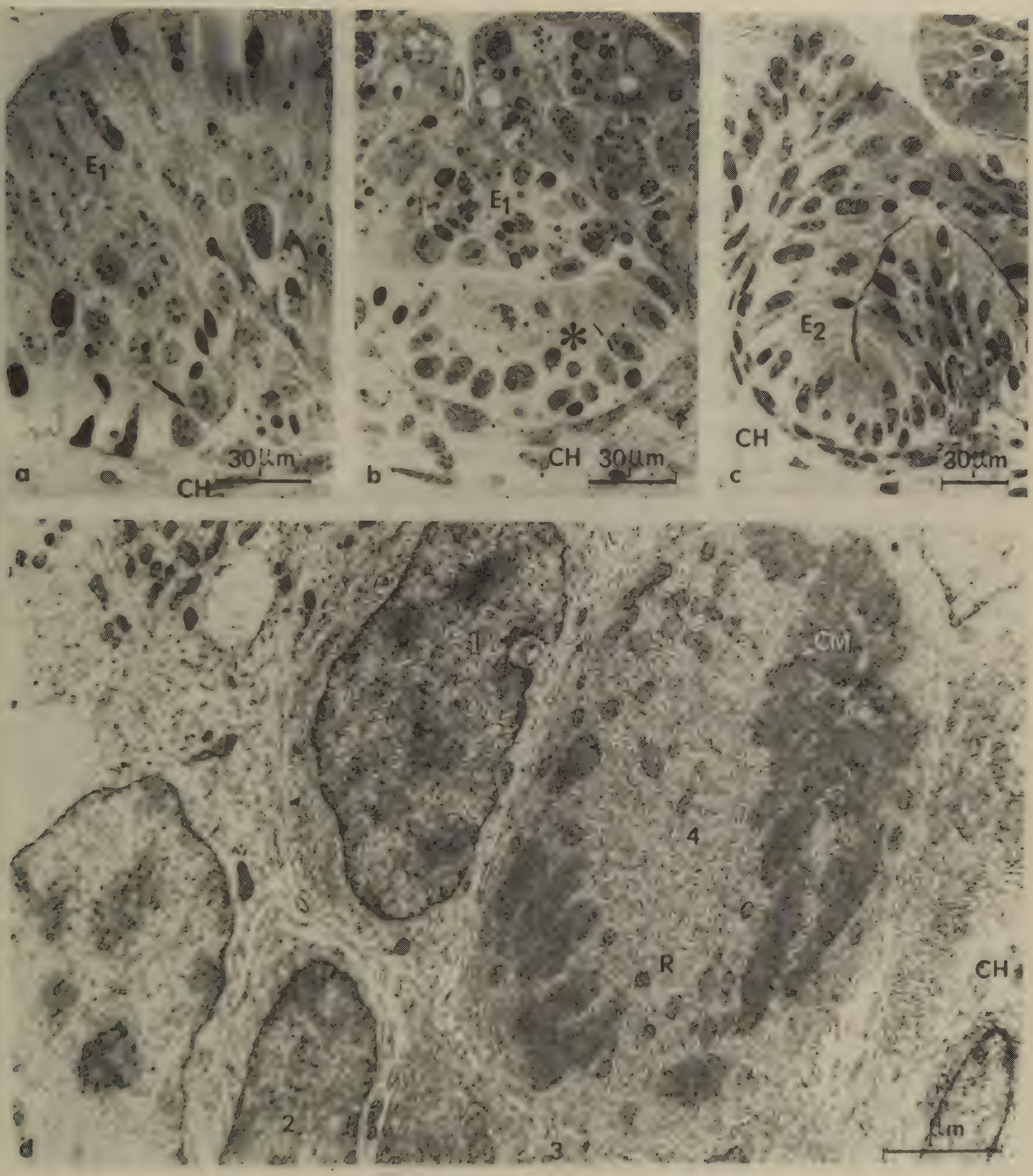


FIGURE 3. — Paroi intestinale de la larve du Discoglosse.

a-c) Coupes semi-fines.

*a*) Prométamorphose (stade XVIII). Deux cellules-souches des épithéliocytes secondaires (flèche) sont localisées à la base de l'épithélium primaire ($E_1$) ; × 550 ;

*b*) Débu: du climax (stade XX). Les épithéliocytes secondaires prolifèrent en formant un îlot (astérisque) sous l'épithélium primaire en dégénérescence ($E_1$) ; × 500.

*c*) Fin du climax (stade XXIII). L'épithélium secondaire est très développé et villeux ($E_2$) ; × 400.

*d*) Début du climax (stade XXI). Détail d'un îlot montrant des épithéliocytes secondaires en voie de prolifération (1-4). Une cellule en mitose est aperçue (4) ; × 21 000.

*CH :* chorion ; *CM :* chromosome ; *R :* ribosome.

Tétroxyde d'osmium-tampon cacodylate.

lisé directement par observation des coupes au microscope optique. Ces mesures sont complétées par une analyse qualitative à l'échelle ultrastructurale.

Durant la prémétamorphose et la prométamorphose, la plupart des cellules appartiennent au seul épithélium primaire (Fig. 3*a*).

Au début du climax, le pourcentage des épithéliocytes primaires en place s'abaisse en relation avec leur dégénérescence et l'évacuation des débris dans la lumière. Les épithéliocytes secondaires nés des cellules souches deviennent plus nombreux et s'agencent en îlots (Fig. 3*b*). Leurs noyaux contiennent un ou deux nucléoles volumineux. Les figures mitotiques sont fréquentes (Fig. 3*b* et *d*). L'examen au microscope électronique confirme le caractère embryonnaire de ces cellules dont le rapport nucléoplasmique est élevé, les organites cytolasmique peu ou pas différenciés, les ribosomes libres abondants (Fig 3*d*).

A la fin du climax, les îlots fusionnent et forment un épithélium secondaire villeux, se substituant à l'ancien (Fig. 3*c*). Les mitoses restent fréquentes à la base des villosités, mais elles deviennent plus rares dès la phase juvénile que suit le climax.

### III. *Résultats radioautographiques après incorporation de thymidine tritiée*

Les radioautographies à l'échelle optique et électronique, relatives aux larves d'Alyte et de Discoglosse, ont permis une exploitation qualitative. Chez les larves *d'Alytes obstetricans*, les radioautographies fournies par les coupes semi-fines, ont été également utilisées pour l'obtention de résultats quantitatifs, et la vitesse de prolifération cellulaire a été appréciée dans chaque zone par le calcul de l'indice de marquage. Ce paramètre exprime le pourcentage de noyaux marqués dans chacune des zones. Sa valeur moyenne, par période, est déterminée en utilisant 6 coupes semi-fines par animal (Fig. 4).

Durant la prémétamorphose, le précurseur est essentiellement localisé dans les noyaux allongés et parallèles de l'épithélium primaire (Fig. 5*a* et *b*), et l'indice de marquage relatif à ce tissu a une valeur moyenne de 8 %. Les cellules souches basales sont rares, peu visibles encore, et leurs noyaux non marqués. Enfin l'indice moyen ne dépasse pas 1 % dans la zone extra-épithéliale réduite à une frange très mince.

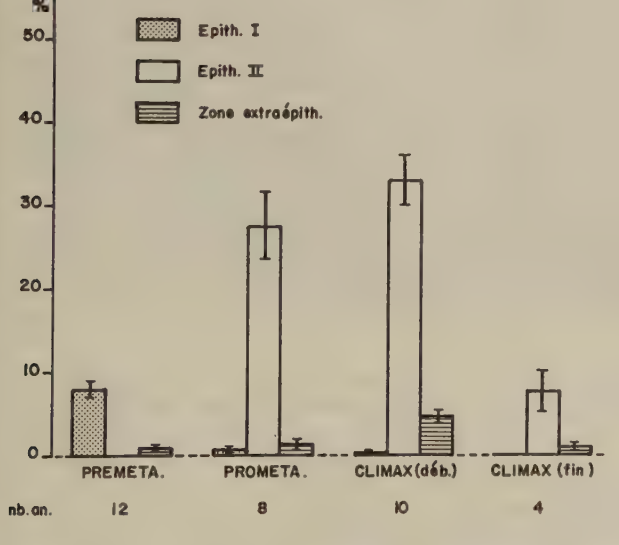


FIGURE 4. — Indice de marquage correspondant à chaque zone de la paroi intestinale, avant et pendant la métamorphose de la larve d'Alyte (en pourcentages moyens ± erreurs-standard).

Au cours de la prométamorphose, les noyaux incorporant la thymidine tritiée sont moins nombreux dans l'épithélium primaire. L'indice de marquage chute pour cette assise et devient très souvent inférieur à 1 %. Les cellules de ce tissu arrêtent de se diviser et commencent à présenter des signes d'altération flagrants, traduits par une fréquence de plus en plus grande des vacuoles autolytiques (Fig. 5*c*). En fin de prométamorphose, le précurseur se localise préférentiellement dans les noyaux des épithéliocytes secondaires qui commencent à se diviser. Leur indice moyen avoisine alors 27 %.

Au début du climax, les radioautographies révèlent un marquage quasi nul au niveau de l'épithélium primaire. Ces résultats sont compatibles avec l'installation de processus autolytiques (Fig. 5*d*) entraînant la dégénérescence puis l'élimination de ce tissu dans la lumière intestinale. Durant cette période, les épithéliocytes secondaires se divisent activement, engendrent des îlots d'abord isolés (Fig. 5*e*) qui refoulent l'épithélium primaire vers la lumière. Ces îlots deviennent ensuite jointifs et forment une assise continue entre la membrane basale et l'épithélium primaire (Fig. 5*d*). Si le marquage affecte de nombreux noyaux (Fig. 5*e* et 6), il n'est pas aperçu au niveau des figures mitotiques. Le temps séparant l'injection du précurseur et le sacrifice (2 heures) est en effet inférieur à la durée de la phase de croissance intercalée entre la phase de synthèse d'ADN et la mitose. Le marquage obtenu correspond par conséquent à une seule génération de cellules. L'indice moyen est ici de l'ordre de 33 %. L'incorporation de thymidine tritiée augmente au début du climax dans les noyaux de la zone extra-épithéliale. Cette dernière s'épaissit par légère hyperplasie au niveau du chorion, de la musculeuse et de l'adventice. L'indice moyen de marquage n'excède pas 5 %.

A la fin du climax, l'épithélium primaire a complètement disparu. L'épithélium secondaire devient villeux (Fig. 5*f*). Son indice moyen s'abaisse au voisinage de 8 %. Il est à remarquer que ses noyaux marqués sont parfois localisés à la base des villosités. Les noyaux de la zone extra-épithéliale cessent rapidement d'incorporer, l'indice moyen correspondant avoisinant celui que l'on obtient en prémétamorphose.

Soulignons que les indices ne diffèrent pas significativement dans une même zone, lorsque l'on examine plusieurs coupes choisies à divers niveaux d'un même intestin dont le point de rebroussement et les spires.

### IV. *Variations de l'activité spécifique de l'ADN*

Les résultats moyens par période sont consignés dans le Tableau 1. Ils reflètent les variations de la vitesse de synthèse de l'ADN intestinal chez les larves d'Alyte.

L'activité spécifique est élevée durant la prémétamorphose mais diminue de manière significative pendant la prométamorphose ($p < 0{,}01$). Elle augmente à nouveau au climax ($0{,}02 < p < 0{,}05$), sans atteindre la valeur calculée en prémétamorphose.

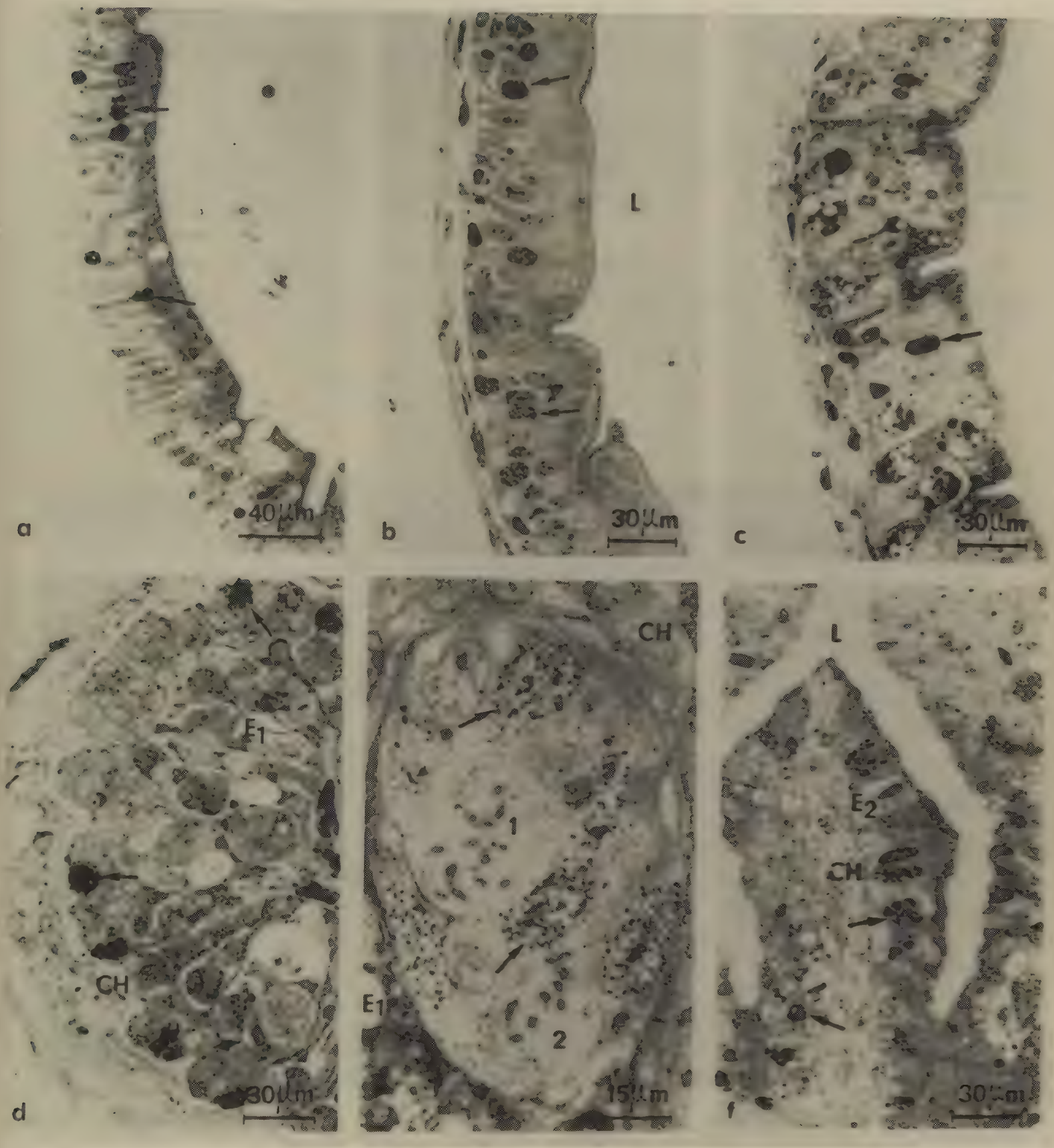


TABLEAU 1. — Activité spécifique de l'ADN intestinal, avant et pendant la métamorphose de la larve d'Alyte.

| Période | Nombre de dosages | Activité spécifique ± Erreur standard |
|---|---|---|
| Prémétamorphose | 8 | 795 ± 161 |
| Prométamorphose | 8 | 194 ± 36 |
| Climax | 10 | 333 ± 51 |

## DISCUSSION

### I. *Evolution des remaniements intestinaux au cours de la métamorphose*

Les résultats précédents permettent de dégager la séquence des événements affectant la paroi de l'intestin, au cours du développement naturel des larves d'Alyte et de Discoglosse.

Durant la prémétamorphose, l'incorporation de la thymidine tritiée est préférentiellement réalisée par les

FIGURE 5. — **Incorporation de la thymidine tritiée par les noyaux des cellules intestinales, avant et pendant la métamorphose.**

a et b) Prémétamorphose (stade X). Les noyaux marqués sont plus spécialement observés dans l'épithélium primaire (*flèches*).

*a*) Discoglosse ; × 350.

*b*) Alyte ; × 400.

*c*) Alyte en prométamorphose (stade XV). Les noyaux marqués sont rares dans l'épithélium primaire (*flèche*) ; × 400.

*d* et *e*) Début du climax (stade XX). L'incorporation du précurseur est réalisée dans les noyaux des épithéliocytes secondaires en îlots (flèches) présents sous l'épithélium primaire altéré ($E_1$).

*d*) Discoglosse ; × 400.

*e*) Alyte. Le marquage est inexistant dans les deux cellules en mitose (1 et 2) ; × 900.

*f*) Alyte en fin de climax (stade XXIV). L'épithélium secondaire ($E_2$) s'accroît et devient villeux. L'incorporation du précurseur est mise en évidence dans plusieurs noyaux, dont quelques-uns sont situés à la base des villosités (*flèches*) ; × 400.

*CH* : chorion ; *L* : lumière intestinale.

*a* et *d* : glutaraldéhyde-tétroxyde d'osmium-tampon cacodylate.

*b*, *c*, *e* et *f* : glutaraldéhyde-tétroxyde d'osmium-tampon phosphate.

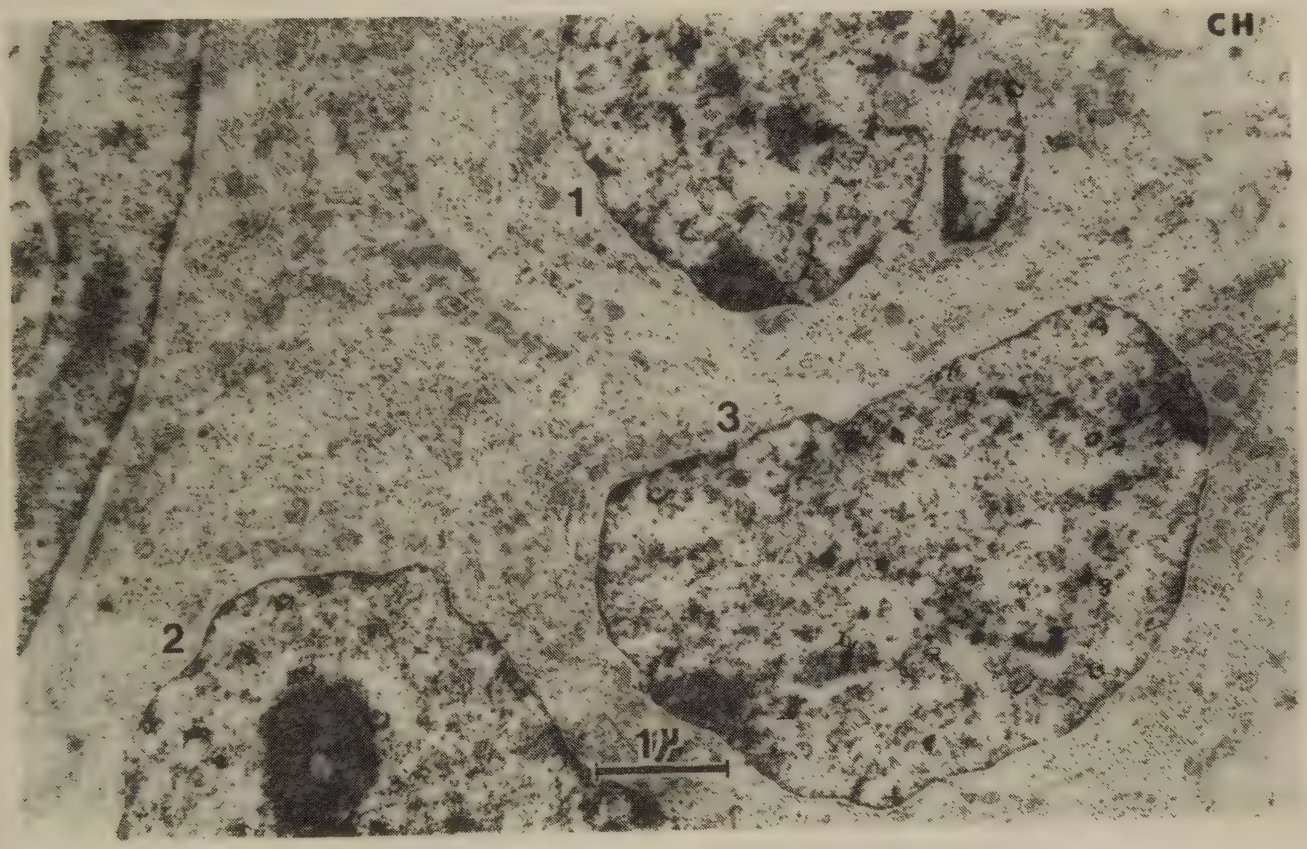


FIGURE 6. — Larve de Discoglosse en début de climax (stade XXI) ayant reçu de la thymidine tritiée. Ilot d'épithéliocytes secondaires dont plusieurs noyaux sont marqués (1-3). *Ch* : chorion. Glutaraldéhyde-tétroxyde d'osmium-tampon cacodylate ; × 18 000.

noyaux des épithéliocytes primaires et, à un moindre degré, par ceux de la zone extra-épithéliale, comme le montre l'examen des indices de marquage. Une telle incorporation ne correspond certainement pas à un simple renouvellement cellulaire. Elle reflète plutôt un processus de croissance de l'intestin, perceptible par l'allongement de l'organe et compatible avec la valeur élevée de l'activité spécifique de l'ADN, que nous obtenons. Cette croissance épithéliale ne semble pas se produire à vitesse constante d'un stade au suivant, étant donné les variations présentées par l'indice.

Au cours de la prométamorphose, une diminution de l'indice de marquage est observée au niveau de l'épithélium primaire et traduit un ralentissement de la croissance du tissu, également remarqué par Dour-

non et Chibon (1974) chez la larve de *Bufo bufo*. Par contre, l'indice relatif aux épithéliocytes secondaires augmente. Ces cellules commencent à se diviser à partir de cellules-souches, aux potentialités replicatives jusqu'alors latentes, mais elles ne constituent encore qu'une très faible partie de la paroi. L'activité spécifique de l'ADN est alors particulièrement faible.

Le début du climax est caractérisé par une dégénérescence de l'épithélium primaire, qui cesse pratiquement d'incorporer. Cette nécrose est provoquée par l'activité d'hydrolases lysosomales diffuses dans le hyaloplasme (Hourdry, 1971*b*). Le matériel altéré est ensuite libéré dans la lumière intestinale. Les épithéliocytes secondaires, nés des cellules-souches prolifèrent activement et engendrent des îlots séparés puis jointifs. L'indice de marquage demeure élevé dans ces îlots, et il avoisine celui que mentionnent Dournon et Chibon (1974) pour l'épithélium intestinal secondaire, chez la larve de *Bufo bufo*. Les indices obtenus au point de rebroussement et à divers niveaux des spires sont en général très proches. Les quelques différences refléteraient une répartition aléatoire des cellules-souches basales plutôt que la propagation d'une onde morphogénétique.

L'indice de marquage est relativement faible dans la zone extra-épithéliale, au moment du climax. La prolifération cellulaire y est toutefois suffisante pour expliquer l'épaississement de la zone.

A la fin du climax, l'épithélium est exclusivement secondaire. Son indice de marquage s'abaisse. Sa croissance se ralentit et, à la phase de prolifération intense, succède une phase de mise en place des villosités. Certaines cellules de la nouvelle assise se transforment en épithéliocytes absorbants, acquérant rapidement des microvillosités apicales (Bonneville et Weinstock, 1970). D'autres deviennent des cellules mucipares caliciformes. Nos observations montrent en outre une fréquence plus élevée des noyaux marqués à la base des villosités et s'accordent avec les résultats d'Hermos (1971), au niveau de l'épithélium intestinal du fœtus de rat, ou d'Ermak (1975) à propos du tractus digestif des Urochordés.

Les processus de croissance que nous constatons dans l'épithélium secondaire et dans la zone extra-épithéliale sont conciliables avec l'activité spécifique accrue de l'ADN, au climax.

Chez les Amphibiens Anoures en métamorphose, la prolifération des cellules-souches basales ne caractérise pas seulement l'épithélium intestinal, puisqu'elle est observée dans l'épithélium gastrique (Forte *et coll.*, 1969 ; Cros et Hourdry, résultats non publiés) et l'épiderme (Wright, 1973). Des cellules embryonnaires analogues ont également été décrites dans l'épithélium gastrique du fœtus de hamster (Arnold, 1966) et dans les disques imaginaux de la drosophile (Hadorn, 1966). Wright (1973) a remarqué une mise au repos temporaire des cellules basales épidermiques du membre postérieur, chez le têtard de *Rana pipiens*, lorsque l'animal est au début de la prométamorphose. Ultérieurement, ces cellules se multiplient et s'organisent en glandes cutanées. Durant cette période de faible incorporation, le métabolisme des cellules basales changerait d'orientation, et les cellules deviendraient aptes à former des glandes. De telles transformations métaboliques sont peu probables au niveau des cellules-souches basales de l'épithélium intestinal. Ces cellules acquièrent en fait très tôt l'ensemble des potentialités leur permettant d'engendrer des épithéliocytes secondaires, comme le montrent les résultats obtenus chez les larves de Discoglosse en prémétamorphose traitées par la thyroxine (Hourdry, 1971*c*).

### II. *Répartition numérique des noyaux marqués entre les trois zones*

L'indice de marquage ne permet pas de savoir quelle fraction de l'ADN nucléaire néosynthétisé revient à chaque zone. L'importance de chacune des fractions est reflétée, sur coupe, par la répartition numérique des noyaux marqués dans les trois zones. Cette répartition est appréciée en rapportant le nombre de noyaux marqués par zone au nombre total des noyaux marqués dans la section. Les coupes semi-fines qui ont servi au calcul de l'indice de marquage sont utilisées. Les valeurs sont exprimées par des pourcentages moyens pour chaque période (Fig. 7).

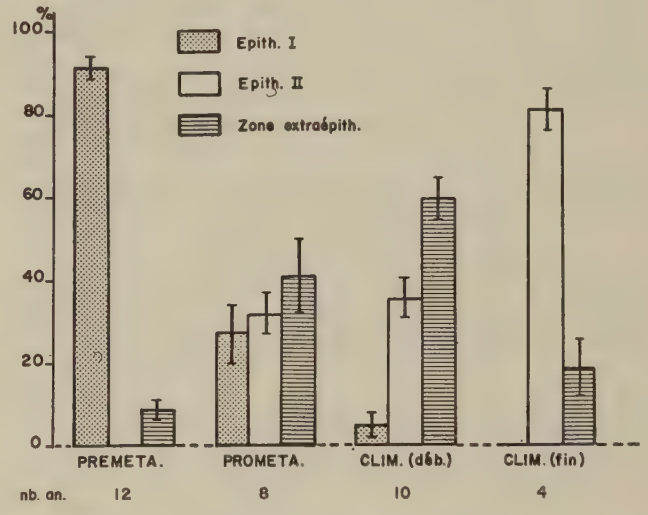


FIGURE 7. — Répartition numérique des noyaux marqués entre les différentes zones de la paroi intestinale, avant et pendant la métamorphose de la larve d'Alyte (en pourcentages moyens ± erreurs-standard).

Dans ces conditions, au cours de la prémétamorphose, plus de 90 % des noyaux marqués sont situés dans l'épithélium primaire.

Pendant la prométamorphose, les cellules-souches des épithéliocytes secondaires commencent à se diviser mais restent rares. Les noyaux secondaires marqués forment alors le tiers de la population particulièrement faible des noyaux marqués pariétaux.

Au début du climax, les noyaux ayant incorporé le précurseur augmentent en nombre, et ils sont aperçus en presque totalité dans l'épithélium secondaire et dans la zone extra-épithéliale. Jusqu'à la fin du climax, le pourcentage de ces noyaux ne cesse de s'élever dans l'épithélium secondaire, lequel devient de plus en plus volumineux.

## CONCLUSION

L'utilisation conjointe des techniques radioautographique et de dosage a permis d'apprécier la localisation et l'intensité de la néosynthèse de l'ADN intestinal, après incorporation de thymidine tritiée par les larves. Les résultats ont mis en relief une évolution différente des trois composantes de la paroi intestinale. L'épithélium primaire dégénère. Un épithélium secondaire le remplace au climax, par prolifération de cellules-souches. Enfin, la zone extra-épithéliale s'épaissit, mais ses cellules se multiplient avec une fréquence modérée. L'antagonisme que nous observons au niveau épithélial, entre une assise primaire et une assise secondaire, doit être souligné. En effet, si l'on met à part l'épiderme de la région operculaire (Kaltenbach, 1968), les phénomènes histolytiques et histogénétiques s'installent le plus souvent dans des organes différents chez les larves d'Anoures.

Le contrôle de l'évolution intestinale est réalisé par les hormones thyroïdiennes dont le taux s'élève dans le plasma dès la prométamorphose (Etkin, 1968 ; Leloup et Buscaglia, 1977 ; Regard et Taurog, 1977). Après traitement par la thyroxine, des remaniements analogues ont été obtenus et quantifiés chez des larves d'Anoures en prémétamorphose (Hourdry, 1971*c* ; Dauça et Hourdry, à publier). Les hormones pourraient provoquer une synthèse d'hydrolases lysosomales, responsables de la nécrose de l'épithélium primaire (Brown et Millington, 1968 ; Hourdry, 1974). Inversement, elles stimulent l'activité duplicative des cellules-souches basales. Cette réponse spécifique des épithéliocytes intestinaux suggère une action des hormones thyroïdiennes sur les mécanismes génétiques, déjà démontrée dans les cellules hépatiques, caudales ou dans les globules rouges (Weber, 1969 ; Cohen, 1970 ; Frieden et Just, 1970 ; Tata, 1972).

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EFFETS IN VIVO ET IN VITRO DE LA THYROXINE SUR L'EPITHELIUM INTESTINAL DES AMPHIBIENS ANOURES.

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# EFFETS IN VIVO ET IN VITRO DE LA THYROXINE SUR L'ÉPITHÉLIUM INTESTINAL DES AMPHIBIENS ANOURES

Michel DAUÇA, Jacques HOURDRY et Jean-Claude POUYET.
Laboratoire de Biologie-Vertébrés, Centre d'Orsay, Université de Paris-Sud, 91405-Orsay.

La métamorphose des Amphibiens Anoures est caractérisée par une série de transformations morphologiques, histologiques et biochimiques (FRIEDEN et JUST, 1970). Plusieurs organes régressent (queue, branchies,...). D'autres se développent (poumons,...). L'intestin tient une place à part, puisque son épithélium est à la fois le siège de phénomènes histolytiques et histogénétiques; simultanément, l'organe diminue de volume et se déspiralise (HOURDRY, 1972).

Les évènements de la métamorphose sont provoqués par une élévation du taux des hormones thyroïdiennes dans le milieu intérieur. Selon ETKIN (1968) la concentration de la thyroxine circulante est multipliée par 100 chez les larves arrivant au paroxysme du phénomène; elle atteindrait alors $10^{-7}$g/l.

Nous avons reproduit expérimentalement les modifications de l'épithélium intestinal.

1°) in vivo, en induisant une métamorphose chez des larves traitées par la thyroxine.

2°) in vitro, en plaçant des fragments d'intestin dans un milieu de culture additionné de l'hormone.

## I. - EFFETS IN VIVO DE LA THYROXINE SUR L'ÉPITHÉLIUM INTESTINAL

Ils sont analysés chez des larves de Discoglosse ou d'Alyte en prémétamorphose, placées dans une solution à 20° de thyroxine à $10^{-7}$g/l (HOURDRY, 1972).

### A. PHÉNOMÈNES HISTOLYTIQUES (Discoglosse).

L'épithélium initial ou primaire dégénère. Dans les cellules en début d'altération, on observe de petites vacuoles autolytiques, ainsi que leurs formes résiduelles, denses et myéliniques. L'accentuation de la dégénérescence est caractérisée par l'apparition de vacuoles autolytiques et de corps résiduels plus grands. L'altération cytoplasmique est parfois accompagnée d'une rétraction. Les cellules les plus dégénérées sont éliminées dans la lumière intestinale. Le nombre d'épithéliocytes altérés augmente au fur et à mesure que le traitement se prolonge. Au bout de 6 jours, l'épithélium primaire est complètement détruit.

Beaucoup de phénomènes dégénératifs sont provoqués par l'activité d'hydrolases lysosomales, agissant de manière optimale à pH acide (DE DUVE et WATTIAUX, 1966). Nous avons recherché l'activité de l'une d'entre elles, la phosphatase acide, lors de la métamorphose induite de l'intestin. L'activité de cette enzyme s'accroît dans l'épithélium primaire avec la durée du traitement. Le produit de réaction est observé dans les vacuoles autolytiques, leurs formes résiduelles, ainsi que dans le hyaloplasme. L'activité spécifique de la phosphatase acide totale, en µM de p-nitrophénol par mg de protéines totales, passe par un maximum lorsque les larves sont traitées de 3 à 6 jours. Elle est alors 3 fois plus élevée que chez les témoins non soumis à l'action hormonale.

### B. PHÉNOMÈNES HISTOGÉNÉTIQUES (Discoglosse et Alyte).

Lorsque le traitement des larves dépasse 3 jours, des cellules embryon-

naires se multiplient activement à la base de l'épithélium primaire. Elles engendrent des îlots qui fusionnent en formant un épithélium secondaire. Celui-ci refoule l'assise primaire en lyse vers l'axe de l'intestin.

Des observations radioautographiques sont réalisées chez des larves ayant reçu de la thymidine tritiée par injection (Discoglosse : 6 µCi; Alyte : 24 µCi; activité spécifique : 15 à 26 µCi/mM). L'indice de marquage des noyaux secondaires accuse un maximum après un traitement hormonal de 4 jours chez le Discoglosse ou de 9 jours chez l'Alyte. L'activité spécifique de l'ADN, en cpm par mg d'ADN, culmine également à 9 jours chez l'Alyte (DAUÇA et HOURDRY, en préparation).

## II. - EFFETS IN VITRO DE LA THYROXINE SUR L'ÉPITHÉLIUM INTESTINAL

Des explants d'intestin de larves de Discoglosse sont placés dans les milieux de Wolf-Quimby ou L15 de Leibovitz additionnés de thyroxine ($10^{-7}$ g/l). Ces milieux de culture sont maintenus à 22°C et oxygénés par agitation rapide. La durée du traitement est comprise entre 1 et 6 jours.

Le nombre des épithéliocytes primaires altérés s'accroît si le traitement se prolonge. Les fragments témoins ne montrent aucun signe de nécrose.

Le mode de dégénérescence observé est très semblable à celui que l'on remarque *in vivo* (POUYET et HOURDRY, en préparation). Des lysosomes nombreux et de dimensions variables apparaissent dans le cytoplasme. Il s'agit de vacuoles autolytiques et de leurs formes résiduelles. Les dictyosomes et les vésicules golgiennes sont fréquents dans les épithéliocytes en cours d'altération. Ces vésicules sont particulièrement abondantes à l'intérieur des lysosomes.

L'activité d'une hydrolase lysosomale, la phosphatase acide, s'élève dans les épithéliocytes en voie de nécrose. Le produit de réaction précipite dans les saccules golgiens, les vésicules qui s'en détachent et les lysosomes. Il se dépose parfois dans le hyaloplasme. Ajoutons que les hydrolases localisées dans les vésicules golgiennes, véritables lysosomes d'accumulation, peuvent largement contribuer à la dégénérescence du contenu des vacuoles autolytiques.

## III. - MODE D'ACTION DES HORMONES THYROIDIENNES

### A. ACTION DIRECTE ET REPONSE SPECIFIQUE.

Une action directe de la thyroxine est démontrée au niveau de différents organes d'Amphibiens (revue dans KALTENBACH, 1968), dont l'intestin (BARRY, 1952, LIPSON, 1965), par implantation locale de pastilles de cholestérol enrichies en hormone. Les altérations que nous apercevons dans les épithéliocytes primaires, à l'issue d'un traitement *in vitro* par la thyroxine, confirment un tel effet.

Les résultats relatifs à la métamorphose induite soulignent la réponse spécifique à la thyroxine de 2 populations d'épithéliocytes intestinaux, puisque les uns dégénèrent selon un processus d'autolyse, alors que les autres se multiplient activement.

### B. SITES D'ACTION.

A l'heure actuelle, ces sites ne sont pas connus pour les épithéliocytes intestinaux des larves d'Anoures. Des résultats particulièrement intéressants sont obtenus à propos du foie et de la queue. Au moment de la métamorphose, le premier organe subit une réorientation métabolique, en relation avec l'apparition de l'uréotélie; le second dégénère. Une liaison de la triiodo-

thyronine à des protéines acides cytoplasmiques est démontrée (YOSHIZATO et coll., 1975a). Ces sites auraient toutefois une faible affinité pour l'hormone. L'existence de sites nucléaires, ayant une haute affinité mais une capacité réduite d'accueil pour les hormones thyroïdiennes, est mise en évidence (YOSHIZATO et coll., 1975b; KISTLER et coll., 1975). La capacité maximale de liaison nucléaire pour la triiodothyronine est multipliée par 2 pendant la métamorphose, dans les noyaux de la queue (YOSHIZATO et FRIEDEN, 1975).

C. MÉCANISME D'ACTION.

Il est essentiellement précisé aux niveaux hépatique et caudal.

L'installation des hormones thyroïdiennes sur les sites nucléaires hépatiques permet l'élaboration d'une nouvelle information génétique, avec stimulation des processus de transcription et de traduction (FRIEDEN et JUST, 1970; COHEN, 1970; TATA, 1972). Une activation précoce de la synthèse des ARN nucléaires et, essentiellement, de l'ARN ribosomal est démontrée dans les hépatocytes. Ultérieurement, la concentration des ARN cytoplasmiques augmente. La synthèse des phospholipides et des protéines de structure s'accélère. Elle conduit à la mise en place de profils de réticulum endoplasmique granulaire (BENNETT et coll., 1970) et à l'apparition de protéines nouvelles, dont les enzymes du cycle de l'urée.

Lors de l'involution caudale, le mécanisme d'action des hormones thyroïdiennes est précisé par l'utilisation d'inhibiteurs de protéosynthèse, comme l'actinomycine D (EECKHOUT, 1969; WEBER, 1969; FRIEDEN et JUST, 1970; TATA, 1972). En empêchant la synthèse des ARN, ce dernier supprime la production des hydrolases responsables de la destruction des tissus caudaux.

Au niveau intestinal, nos connaissances sont encore très fragmentaires. In vivo ou in vitro, un début d'organisation du réticulum granulaire est aperçu dans la zone paranucléaire des épithéliocytes primaires, lorsque le traitement par la thyroxine est inférieur à 3 jours. Ces observations sont compatibles avec une synthèse accrue d'enzymes lysosomales. Certaines, dont la phosphatase acide, pourraient subir une glycosylation supplémentaire en transitant par les dictyosomes et les nombreuses vésicules golgiennes (GOLDSTONE et KOENIG, 1973). Un contrôle hormonal de la perméabilité lysosomale est également suggéré par l'existence d'une activité phosphatasique acide, diffuse dans le hyaloplasme.

Un effet accélérateur des hormones thyroïdiennes sur la duplication de l'ADN est prouvé par l'élévation de l'indice de marquage des noyaux secondaires. De nouvelles enzymes digestives apparaissent dans l'épithélium secondaire, en relation avec un changement de régime alimentaire. Elles supposent une modification de l'activité génique par les hormones, retentissant sur les vitesses de transcription et de traduction.

L'activation des mécanismes génétiques, propres aux épithéliocytes primaires et secondaires de l'intestin, n'est pour le moment qu'une simple hypothèse de travail. Nos recherches actuelles s'inscrivent dans son cadre.

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# ANALYSE QUANTITATIVE DES REMANIEMENTS INTESTINAUX ET EVOLUTION DE LA SYNTHESE DE L'ADN.

Etude chez les larves d'*Alytes obstetricans* (Amphibien Anoure) traitées par la thyroxine.

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# Analyse quantitative des remaniements intestinaux et évolution de la synthese de l'ADN

## Etude chez les larves d'*Alytes obstetricans* (Amphibien Anoure) traitées par la thyroxine

M. Dauça et J. Hourdry

Laboratoire de Biologie-Vertébrés, Centre d'Orsay, Université Paris-Sud, F-91405-Orsay, France
Laboratoire de Sciences Naturelles de l'Ecole Normale Supérieure de Saint-Cloud, 2, avenue du Palais, F-92211-Saint-Cloud, France

## Quantitative Analysis of Intestinal Changes and the Evolution of DNA Synthesis in Thyroxine-Treated Larvae of *Alytes obstetricans*. (Amphibia, Anura)

**Summary.** Changes in the intestinal wall of *Alytes obstetricans* (Amphibia, Anura) were quantified during thyroxine treatment. The relative proportion of each intestinal wall component was determined in tissue sections. Autoradiographic studies on intestine with $^3$H-thymidine revealed the distribution and frequency of labeled nuclei.

The labeling index in the primary epithelium falls to zero by 3 days. Thyroxine treatment induces a decrease in the growth rate of this tissue, just before its degeneration and complete elimination by 14 days.

Three days after $T_4$-treatment, a secondary epithelium develops from basal stem cells. Its labeling index rises to a maximum of about 50% by 9 days, and thereafter declines. Cell proliferation is less marked in the extra-epithelial zone, which nevertheless thickens during this thyroxine-induced metamorphosis.

**Key words:** Tadpole – Intestine – Labeling index – Thyroxine – Cell proliferation.

**Résumé.** Les remaniements intestinaux affectant la paroi intestinale sont quantifiés chez les larves d'un Amphibien Anoure, *Alytes obstetricans*, traitées par la thyroxine. La fraction correspondant à chaque zone composant la paroi est déterminée sur coupe. La répartition et la fréquence des noyaux marqués sont recherchées chez des larves ayant reçu de la thymidine tritiée.

L'indice de marquage nucléaire de l'épithélium primaire tend vers zéro durant les 3 premiers jours du traitement. Ces résultats reflètent un ralentissement de la croissance du tissu, précédant sa dégénérescence et son élimination complète au 14ème jour d'immersion dans la solution hormonale.

Dès le 3ème jour de traitement, un épithélium secondaire se développe à partir de cellules-souches basales. Son indice de marquage croît, culmine au 9ème jour avec une valeur proche de 50%, puis s'abaisse. La prolifération cellulaire est plus modérée dans la zone extra-épithéliale, qui s'épaissit toutefois au cours de cette métamorphose provoquée.

Depuis les travaux de Gudernatsch (1912), il est classiquement admis que la métamorphose des Amphibiens Anoures est provoquée par les hormones thyroïdiennes. L'augmentation présumée de la concentration de ces hormones dans le plasma (Etkin, 1968; Just, 1972) entraîne l'apparition de spectaculaires transformations morphologiques qui affectent la plupart des organes dont l'intestin. Ce dernier se déspiralise et se raccourcit, tandis que son épithélium dégénère et est remplacé par une assise de néoformation (Bonneville, 1963; Hassan et Marinucci, 1966; Hourdry, 1969a, b, 1971a; Kopéc, 1970; Dournon et Chibon, 1974).

Des remaniements intestinaux analogues sont obtenus chez les larves d'Anoures, après traitement par la thyroxine (Hourdry, 1971b) ou, in vitro, avec des explants placés dans un milieu de culture additionné ou non d'hormone (Delsol, 1966; Pouyet et Hourdry, sous presse).

L'analyse quantitative des transformations intestinales fait l'objet du présent travail. Une première approche est réalisée sur coupes semi-fines, en mesurant la surface de chaque composant de la paroi et en pratiquant des numérations cellulaires. Une seconde investigation est effectuée après incorporation de thymidine tritiée. Elle consiste en une analyse radioautographique de la fréquence et de la répartition des noyaux marqués, synthétisant de l'ADN avant de se diviser.

## Matériel et méthodes

Les larves d'Alyte sont choisies à des stades précoces de la prémétamorphose, alors que les membres postérieurs sont réduits à des bourgeons (stade I–V de la table de Taylor et Kollros, 1946). Elles sont immergées de 1 à 18 jours dans une solution de DL thyroxine (Roche) à la concentration de $10^{-7}$ g/ml. Les larves sont régulièrement nourries à l'aide de salade cuite. La solution hormonale est renouvelée tous les deux jours et maintenue à la température de 20° C ± 1° C. Des larves de même stade sont laissées dans l'eau du robinet et servent de témoins. La mortalité des larves séjournant dans la solution hormonale est fonction de la durée du traitement: nulle après 24 h d'immersion, elle peut atteindre 10 à 20% au bout de 18 jours.

Deux heures avant leur sacrifice, les larves reçoivent 24 µCi de thymidine tritiée par injection intrapéritonéale (activité spécifique: 15 à 26 Ci/mM).

L'intestin est ensuite prélevé et soumis, pendant 25 min à 4° C, à une première fixation par le glutaraldéhyde à 2% dans le tampon phosphate de Sörensen (0,15 M). Dans ce mélange dont le PH est ajusté à 7,4 l'organe est vidé et débité transversalement en petits fragments. Après un lavage rapide dans le tampon, les pièces sont post-fixées une heure, à froid, dans un mélange volume à volume de tétroxyde d'osmium (2%) et de tampon phosphate. Les fragments d'intestin sont orientés dans le milieu d'inclusion (épon 812), afin d'être coupés transversalement.

Les fragments d'intestin sont débités en coupes semi-fines de 2,5 µ d'épaisseur. Les lames portant les coupes sont recouvertes d'une émulsion nucléaire Ilford L4 diluée 3 fois, séchées et placées dans des boîtes hermétiques de clay-Adams, à 4° C pendant 15 à 18 jours, en présence

d'actigel, selon la technique de Larra et Droz (1970). La révélation est réalisée par le D. 19 (Kodak). Les coupes semi-fines sont ensuite lavées, fixées dans une solution de thiosulfate de sodium à 20%, rincées à nouveau et colorées par le mélange bleu de toluidine-pyronine (Ito et Winchester, 1963).

La surface de la paroi et celles des différentes zones composantes sont mesurées à l'aide d'un planimètre (Maho). La numération des cellules appartenant aux épithéliums primaire et secondaire est réalisée par comptage direct des noyaux, à partir des mêmes coupes transversales.

## Résultats

### *1. Effets de la thyroxine sur les divers tissus de la paroi intestinale*

*Variations de la surface de chaque zone.* Sur coupe transversale, la paroi de l'intestin peut se décomposer en 3 zones:

– L'épithélium initial ou primaire.
– L'épithélium de néoformation ou secondaire.
– La zone extra-épithéliale qui comprend le chorion conjonctif, la musculeuse et l'adventice.

Six coupes semi-fines transversales, prises à divers niveaux de l'intestin, sont utilisées par animal. Sur chacune des coupes, la surface de chaque zone composante est exprimée par un pourcentage de la surface de la paroi. Les résultats moyens obtenus pour les différents temps d'exposition à la thyroxine sont indiqués sur la Figure 1.

Chez les larves témoins, l'épithélium primaire forme l'essentiel de la paroi. Au cours du traitement, ce tissu régresse et disparaît au 14ème jour.

Jusqu'au 4ème jour, les épithéliocytes secondaires sont peu nombreux, et leur surface est difficilement mesurable. Ces épithéliocytes, ou les cellules souches

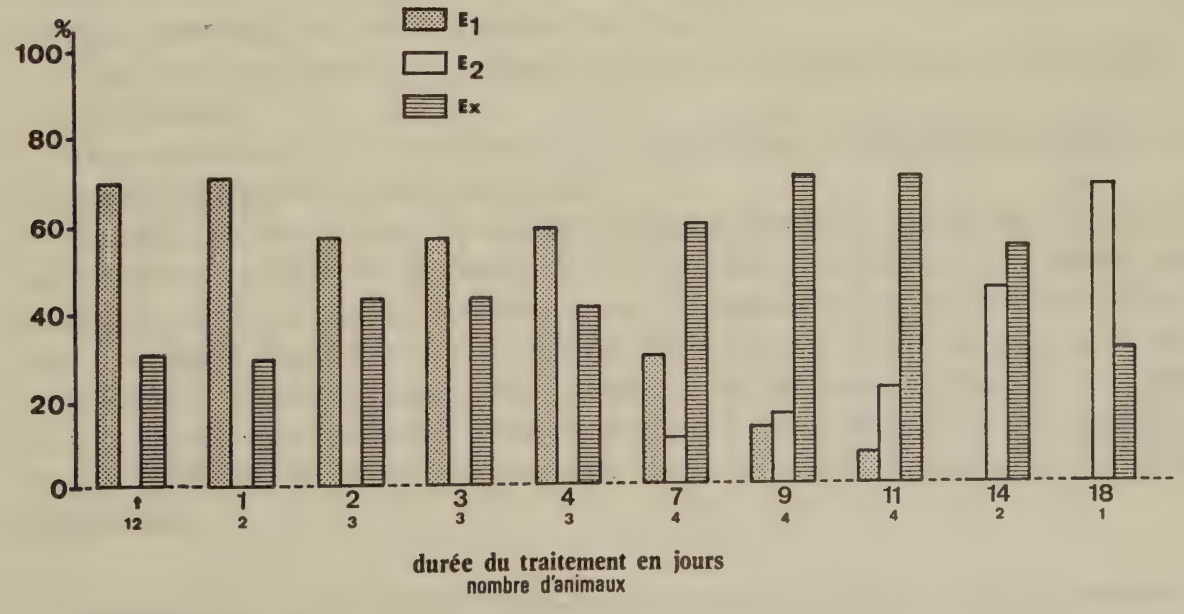


**Fig. 1.** Surfaces des diverses zones de la paroi intestinale, après traitement des larves par la thyroxine (en pourcentages moyens). Abréviations utilisées dans les Figures 1 – 3 et 5: *t* témoin: $E_1$ épithélium primaire: $E_2$ épithélium secondaire: *Ex* zone extra-épithéliale

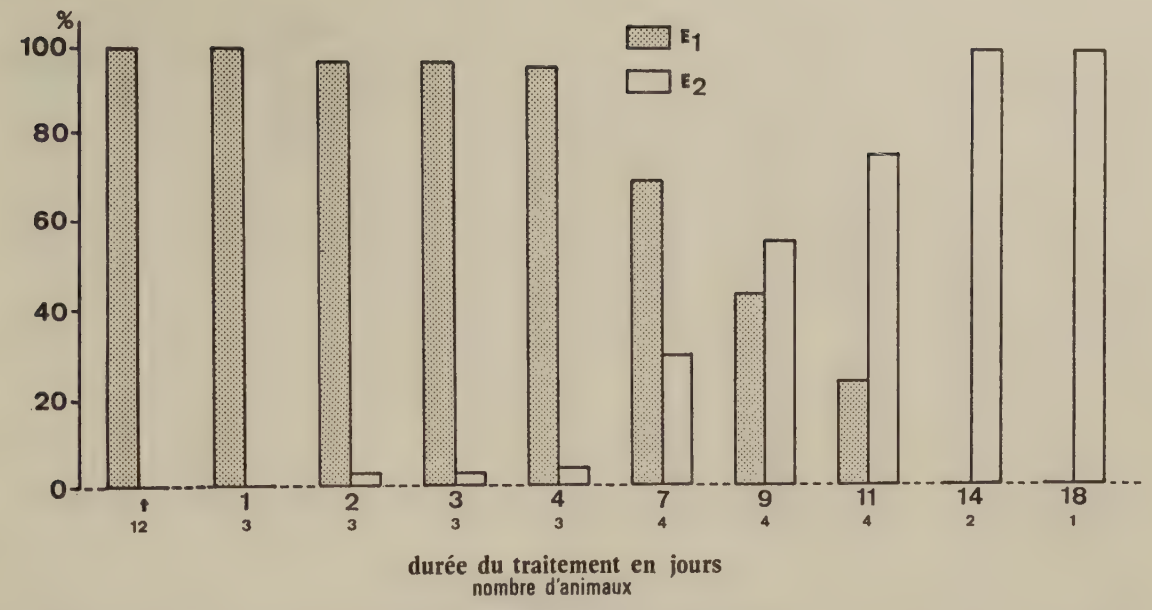


**Fig. 2.** Importances des populations cellulaires dans les épithéliums primaire et secondaire, après traitement des larves par la thyroxine (en pourcentages moyens)

dont ils dérivent, sont localisés à la base de l'épithélium primaire. Lorsque le traitement est prolongé, l'épithélium secondaire s'accroît, perd son aspect en îlots et forme une assise continue et villeuse.

La zone extra-épithéliale s'épaissit fortement, au point de dépasser parfois la moitié de la surface totale.

A ces remaniements, sont associées des modifications d'ordre morphologique, avec raccourcissement et déspiralisation de l'organe.

*Evolution de la population cellulaire dans les deux épithéliums.* Les sections utilisées pour la planimétrie ont également servi à calculer les nombres des épithéliocytes primaires et secondaires, que nous avons exprimés par des pourcentages moyens pour chaque temps d'exposition (Fig. 2).

Chez les larves témoins, la plupart des cellules appartiennent au seul épithélium primaire.

Jusqu'au 4ème jour du traitement, les épithéliocytes primaires montrent des signes d'autolyse mais restent en place.

Au-delà du 4ème jour, les épithéliocytes primaires complètement détruits sont évacués en nombre croissant dans la lumière intestinale. Il en résulte une raréfaction de ces cellules. Simultanément, les épithéliocytes secondaires deviennent nombreux dans les îlots. Leur noyau contient un ou plusieurs gros nucléoles. Leur frange cytoplasmique est très basophile sur coupes semi-fines et l'examen au microscope électronique y révèle de nombreux ribosomes libres. Ces épithéliocytes secondaires finissent par composer la totalité de la population cellulaire épithéliale.

### *2. Incorporation de thymidine tritiée par chaque assise de la paroi intestinale, au cours du traitement par T4*

La vitesse de prolifération cellulaire est appréciée dans chaque zone par le calcul de l'indice de marquage. Ce paramètre est un pourcentage définissant

**Fig. 3.** Indices de marquage correspondant à chaque zone de la paroi intestinale, après traitement des larves par la thyroxine (en pourcentages moyens)

le rapport du nombre de noyaux marqués au nombre total de noyaux, dans chacune des zones. Sa valeur moyenne est déterminée pour chaque durée d'exposition, en utilisant 8 coupes semi-fines par animal (Fig. 3). L'épaisseur des coupes est de 2,5 microns.

Chez les larves témoins, le précurseur est présent dans les noyaux de l'épithélium primaire, où l'indice de marquage atteint une valeur de 8%. Aucun noyau marqué n'est aperçu dans les cellules souches des épithéliocytes secondaires. Enfin, l'indice est inférieur à 1% dans la zone extra-épithéliale.

L'incorporation de la thymidine tritiée par les noyaux de l'épithélium primaire ne cesse de décroître au cours du traitement hormonal (Fig. 4a et b) et, dès le 3ème jour, l'indice de marquage de ce tissu ne dépasse plus 1%.

Si la durée d'exposition à l'hormone est inférieure à 3 jours, les noyaux des cellules souches incorporent rarement le précurseur. A partir du 3ème jour un marquage nucléaire apparaît dans les îlots isolés ou jointifs d'épithéliocytes secondaires (Fig. 4c–e). L'indice croît et culmine chez les têtards maintenus 9 jours dans la solution de thyroxine. Sa valeur est alors proche de 50% et très nettement supérieure à celle que l'on obtient au climax naturel (Dauça et Hourdry, à publier). L'hormone induit par conséquent une prolifération intense des épithéliocytes secondaires.

Lorsque le traitement est prolongé au-delà de 9 jours, l'indice de marquage de l'épithélium secondaire diminue mais ne s'annule pas. Les épithéliocytes poursuivent alors leurs divisions avec une fréquence plus faible, et ils permettent la mise en place de villosités oblitérant la lumière intestinale (Fig. 4f–h). Les foyers de prolifération et les noyaux marqués restent souvent localisés à la base de ces villosités.

L'incorporation de la thymidine tritiée augmente jusqu'au 7ème jour dans la zone extra-épithéliale puis s'abaisse, sans que l'indice de marquage n'atteigne jamais 8%.

Soulignons enfin que les indices ne diffèrent pas significativement dans une

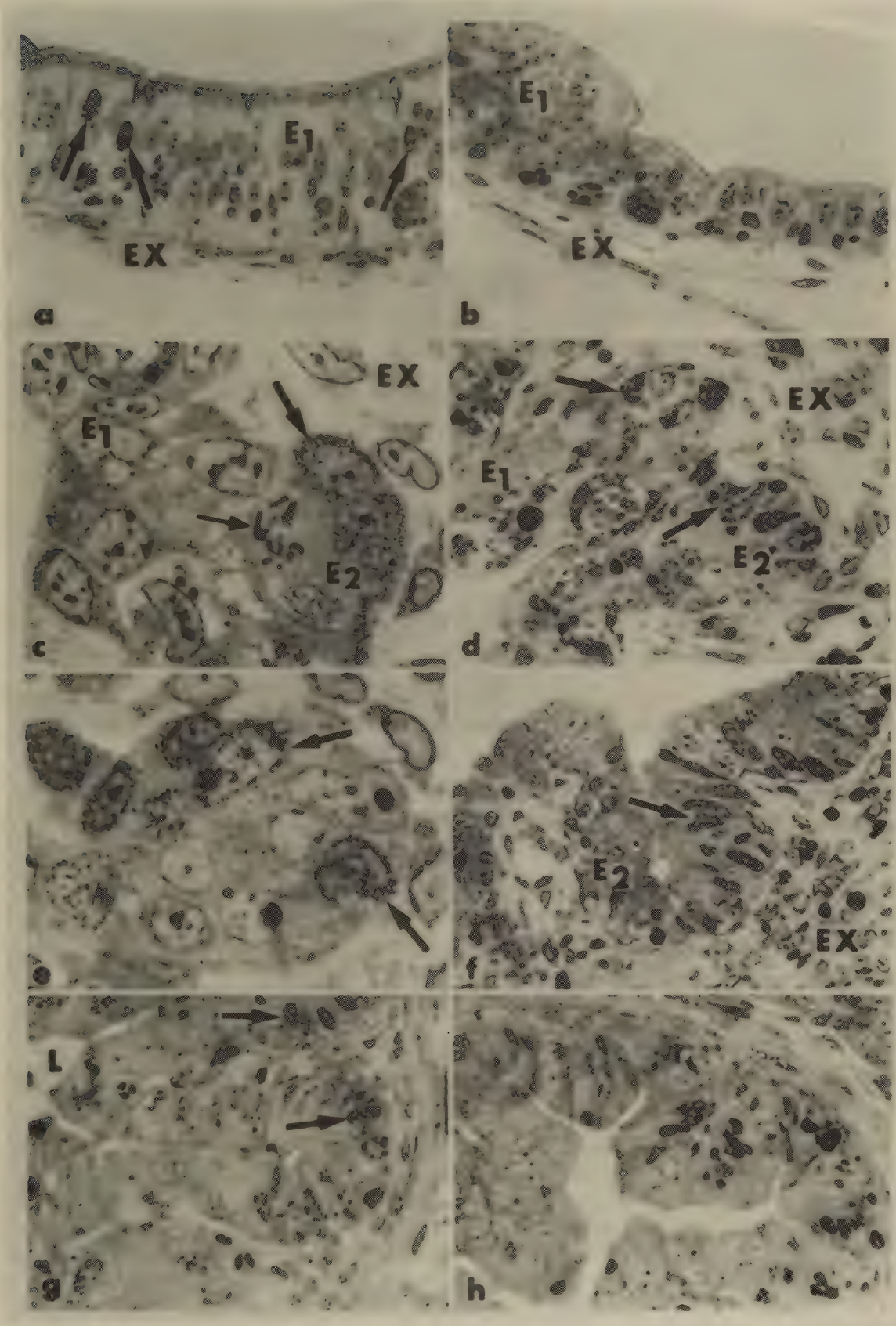


**Fig. 4a – h.** Incorporation de la thymidine tritiée par les noyaux des cellules intestinales, chez les larves traitées par la thyroxine. **a** Traitement de 1 jour. Stade initial IV. Trois noyaux de l'épithélium primaire sont marqués (flèches), et la zone extra-épithéliale est encore très mince 300 ×. **b** Traitement de 4 jours. Stade initial IV. Aucun noyau marqué n'est aperçu dans l'épithélium

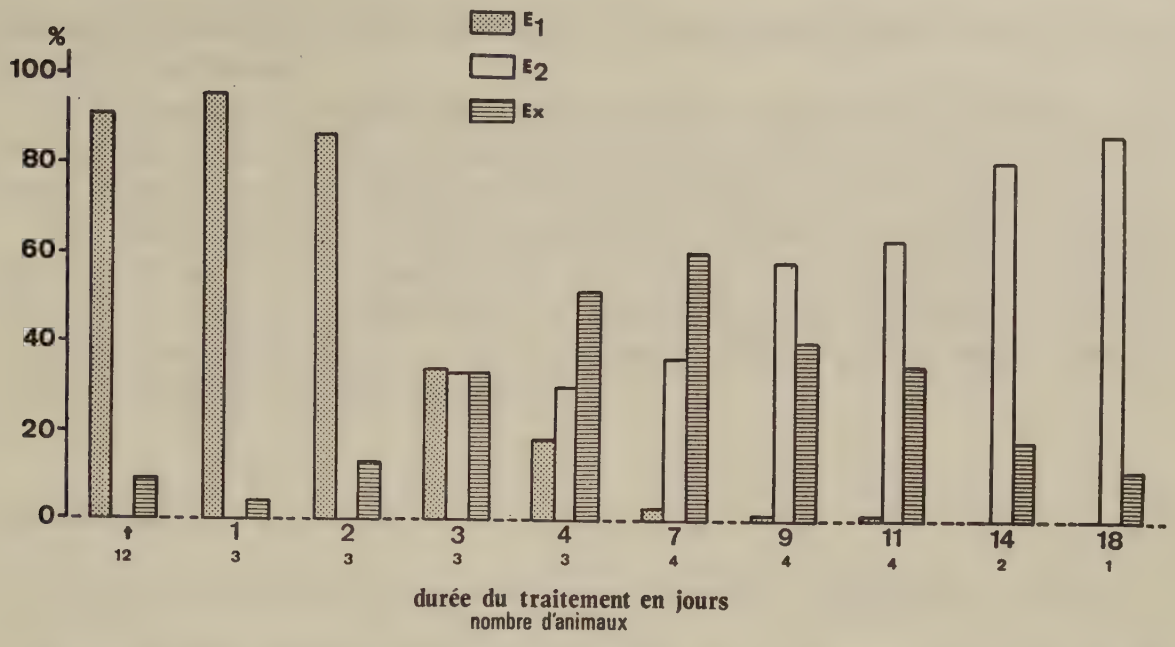


**Fig. 5.** Répartition numérique des noyaux marqués entre les différentes zones de la paroi intestinale, après traitement des larves par la thyroxine (en pourcentages moyens)

même zone, lorsque l'on examine plusieurs coupes prises à des niveaux différents d'un même intestin.

### *3. Répartition numérique des noyaux marqués entre les trois zones*

L'indice de marquage ne permet pas de savoir quelle fraction de l'ADN nucléaire néosynthétisé revient à chaque zone. L'importance de chacune des fractions est reflétée, sur coupe, par la répartition numérique des noyaux marqués dans les trois zones. Cette répartition est appréciée en rapportant le nombre des noyaux marqués par zone au nombre total des noyaux marqués dans la section. Les coupes semi-fines qui ont servi au calcul de l'indice de marquage sont utilisées (Fig. 4a – h). Les valeurs sont exprimées par des pourcentages moyens pour chaque temps d'exposition à la thyroxine (Fig. 5).

Chez les larves témoins, plus de 90% des noyaux marqués sont situés dans l'epithélium primaire. Durant les deux premiers jours du traitement, les noyaux

primaire. 300×. **c** Traitement de 7 jours. Stade initial V. Dans l'îlot, l'un des épithéliocytes secondaires est en mitose (flèche mince), et les autres ont leurs noyaux marqués (flèche large). La zone extra-épithéliale s'est épaissie. 550×. **d** Traitement de 9 jours. Stade initial IV. Sous l'épithélium primaire en cours d'autolyse, les épithéliocytes secondaires forment des îlots jointifs, et la plupart de leurs noyaux sont marqués (flèches). 450×. **e** Traitement de 9 jours. Stade initial IV. Détail d'un îlot d'épithéliocytes secondaires, où les noyaux ont incorporé le précurseur (flèches). 600×. **f** Traitement de 11 jours. Stade initial V. Certains noyaux de l'épithélium secondaire, continu et plissé, sont marqués (flèche). 300×. **g** Traitement de 14 jours. Stade initial V. L'épithélium secondaire oblitère la lumière intestinale par ses villosités, à la base desquelles des noyaux marqués sont plus spécialement aperçus (flèches). 300×. **h** Traitement de 14 jours. Stade initial V. Autre aspect de la paroi. 300×. $E_1$ épithélium primaire; $E_2$ épithélium secondaire en ilôt ou villeux; *Ex* zone extra-épithéliale; *L* lumière de l'intestin. Fixation: Glutaraldéhyde – Tétroxyde d'osmium – Tampon phosphate

marqués deviennent moins nombreux, et ils demeurent essentiellement localisés dans cet épithélium.

Au 3ème jour, les épithéliocytes secondaires commencent à se diviser, mais restent encore rares. Les noyaux marqués de ces cellules forment alors le tiers de la population particulièrement faible des noyaux marqués pariétaux.

Au-delà du 3ème jour, les noyaux ayant incorporé le précurseur, augmentent en nombre, et ils sont aperçus en presque totalité dans l'épithélium secondaire et dans la zone extra-épithéliale. Le pourcentage de ces noyaux ne cesse de s'élever dans l'épithélium secondaire, lequel prolifère avec une relative intensité et devient de plus en plus volumineux.

## Discussion

### *1. Profil des remaniements intestinaux au cours de la métamorphose induite*

La technique radioautographique a été utilisée pour étudier l'effet de la thyroxine sur la prolifération des diverses cellules intestinales d'*Alytes obstetricans*. Avant de se diviser toute cellule entre dans un phase de synthèse d'ADN ou phase S, au cours de laquelle elle est capable d'incorporer les précurseurs de cet acide nucléïque et notamment la thymidine tritiée. Il est important au cours de l'examen des radioautographies de faire la discrimination entre d'une part les noyaux synthétisant de l'ADN et de ce fait marqués par le précurseur radioactif et, d'autre part les noyaux dont le marquage ne correspond qu'à une «synthèse non programmée» en rapport simplement avec une éventuelle «réparation» de la molécule. Dans ce dernier cas, le marquage nucléaire est toujours réduit et diffus. En ce qui concerne les noyaux marqués de la paroi intestinale, la densité des grains y est, d'une façon générale, si forte qu'il n'est pas possible de les dénombrer. La méthode radioautographique permet donc de définir les conséquences de l'action de la thyroxine sur la paroi intestinale:

– Un traitement d'une durée inférieure à un jour n'induit pas de modifications intestinales profondes. Les radioautographies obtenues sont comparables à celles fournies par les témoins.

– Pour un séjour prolongé dans la solution hormonale, l'indice de marquage de l'épithélium primaire ne cesse de diminuer. Ce tissu présente dès le 3ème jour de traitement, d'importants signes d'autolyse dans les épithéliocytes primaires. Leur dégénérescence est provoquée par l'activité d'hydrolases lysosomales diffuses dans le hyaloplasme (Hourdry, 1971 b).

– Après une latence de 3 jours, la thyroxine induit la prolifération des épithéliocytes secondaires. L'indice de marquage de cette assise en néoformation passe au 9ème jour par un maximum voisin de 50%, puis diminue par la suite. Au cours du traitement, l'indice de marquage de la zone extra-épithéliale s'accroît modérément en relation avec son épaississement.

Des observations semblables ont été réalisées lors de la métamorphose spontanée (Dournon et Chibon, 1974; Dauça et Hourdry, à publier), alors que le taux des hormones thyroïdiennes s'élève dans le plasma (Etkin, 1968; Just, 1972).

### *2. Caractères de la réponse à la thyroxine*

Utilisée à une concentration suffisante, la thyroxine peut déclencher les évènements de la métamorphose dès la formation de l'opercule (Etkin, 1950; Moser, 1950). Le moment où l'intestin acquiert cette aptitude à la réponse, ou compétence, est lui-même très précoce. Des remaniements intestinaux ont toujours été observés chez les larves traitées dont le stade ne dépasse jamais le stade V de la table de Taylor et Kollros (1946). L'acquisition de la compétence par les organes nécessiterait un degré minimal de différenciation histologique et la présence de récepteurs hormonaux (Frieden et Just, 1970; Tata, 1970; Etkin et Gona, 1974).

La réponse des tissus intestinaux à la thyroxine apparaît après une certaine latence. Si les premiers signes d'autolyse s'installent dans l'épithélium primaire, après 24 h de traitement (Hourdry, 1971 b), l'épithélium secondaire ne prolifère pas avant 3 ou 4 jours. L'existence d'une latence précédant la réponse a été signalée au niveau du foie (Cohen, 1970; Atkinson et coll., 1972) et de la queue (Weber, 1969). Sa durée est discutée, mais sa seule existence suggère que cette hormone agit indirectement.

Le traitement par la thyroxine stimule la synthèse de l'ADN et accroît l'activité mitotique dans l'oeil, chez les larves d'Anoures (Kaltenbach et Hobbs, 1972). Au niveau du foie, l'action des hormones thyroïdiennes exogènes est plus discutée. Selon Finamore et Frieden (1960), la quantité d'ADN hépatique décroît. Pour d'autres auteurs (Paik et coll., 1961; Cohen, 1970) cette quantité demeure constante et reflète l'existence d'hépatocytes ne se divisant pas. Selon Atkinson et coll. (1972), Kistler et Weber (1975), les hormones stimulent la synthèse de l'ADN par le foie et provoquent une légère hyperplasie. Une dualité des réponses est signalée par Wright (1973) à propos de l'épiderme, après immersion de larves dans une solution de thyroxine. L'auteur constate un arrêt de la multiplication cellulaire dans les assises superficielles, mais il observe une forte activité mitotique dans l'assise profonde. Au niveau de la paroi intestinale des larves traitées, les réponses obtenues sont également spécifiques de chaque zone, puisque l'épithélium primaire dégénère, l'épithélium secondaire prolifère et la zone extra-épithéliale s'accroît modérément. Cette pluralité des réponses suggère l'application de programmes génétiques différents par les cellules de chaque zone, en présence de thyroxine, aboutissant par exemple à la synthèse d'hydrolases lytiques dans l'épithélium primaire et de protéines de structure dans l'épithélium secondaire.

Dans les épithéliocytes secondaires, l'incorporation de la thymidine tritiée est essentiellement réalisée par le noyau et, à un moindre degré par les mitochondries. Ces observations corroborent celles de plusieurs auteurs, selon lesquels les hormones thyroïdiennes stimulent à la fois la synthèse de l'ADN nucléaire et mitochondrial (Campbell et coll., 1969; Paik et Baserga, 1971; Atkinson et coll., 1972).

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VARIATIONS DE L'ACTIVITE SPECIFIQUE DE L'ADN INTESTINAL CHEZ LA LARVE D'*ALYTES OBSTETRICANS* (AMPHIBIEN ANOURE) TRAITEE PAR LA THYROXINE.

Comptes-rendus de l'Académie des Sciences de Paris, 286, 973-975 (1978).

PHYSIOLOGIE COMPARÉE. — *Variations de l'activité spécifique de l'*ADN *intestinal chez la larve d'*Alytes obstetricans *(Amphibien Anoure) traitée par la thyroxine.* Note (*) de **Michel Dauça** et **Jacques Hourdry**, présentée par M. Pierre-Paul Grassé.

Chez les larves d'Alyte en prémétamorphose, le traitement par la thyroxine modifie l'activité spécifique de l'ADN intestinal. L'activité décroît jusqu'au 3e jour puis présente un pic culminant au 9e jour. Ces résultats sont discutés, compte tenu de résultats radioautographiques antérieurs.

*Treatment by thyroxine modified the specific activity of intestinal* DNA *in premetamorphic* Alytes *tadpoles. The activity decreased up to the 3rd day, and showed afterwards a peak which culminates on the 9th day.*

*These results have been discussed in comparison with previous radioautographic observations.*

Les hormones thyroïdiennes ont une action déterminante sur le déclenchement et le déroulement de la métamorphose, chez les Amphibiens Anoures. Le traitement des larves par la thyroxine ($T_4$) provoque une métamorphose anticipée en élevant artificiellement le taux de l'hormone dans le plasma [(1), (2)]. D'autre part, le dosage radioimmunologique a récemment montré que les concentrations des hormones thyroïdiennes s'accroissent au début du climax de la métamorphose naturelle [(3), (4) et (5)], au moment où s'installent les transformations les plus spectaculaires.

Plus particulièrement, le traitement par la $T_4$ a permis de reproduire précocement la métamorphose de l'intestin, au cours de laquelle l'épithélium primaire dégénère et est remplacé par un épithélium secondaire, cependant que la zone extra-épithéliale s'épaissit (6). La cinétique de ces remaniements a été précisée par une analyse radioautographique (7), en déterminant les variations de l'indice de marquage nucléaire dans chaque partie de l'intestin, après incorporation de thymidine tritiée par des larves d'Alyte traitées.

Les variations de l'activité spécifique de l'ADN intestinal ont été recherchées chez des larves d'Alyte exposées à la $T_4$ et ayant reçu la thymidine tritiée.

MATÉRIEL ET MÉTHODES. — Les larves d'Alyte sont choisies en cours de prémétamorphose, aux stades I-IV de la table de Taylor et Kollros (8). Elles sont immergées de 1 à 11 jours dans une solution de DL thyroxine (Roche) à la concentration de $10^{-7}$ g/ml, renouvelée tous les 2 jours et maintenue à 20°C. Des larves de même stade sont laissées dans l'eau de source et servent de témoins.

2 h avant leur sacrifice, les animaux reçoivent 24 µCi de thymidine tritiée par injection intrapéritonéale (activité spécifique : 15 à 26 Ci/mM).

L'extraction de l'ADN total est réalisée selon la méthode de Munro et Fleck (9). Étant donné la petite taille des intestins, chaque extraction est effectuée en utilisant plusieurs organes (6 au maximum).

La concentration de l'ADN total, en microgrammes par millilitre, est déterminée au moyen d'un spectromètre à ultraviolets (Beckman) réglé à la longueur d'onde de 260 nm. La radioactivité contenu dans 0,2 ml de la solution d'ADN est mesurée au compteur à scintillation (Beckman). L'activité spécifique de l'ADN est calculée, compte tenu de la quantité d'ADN présente dans la fraction aliquote, et exprimée en coups par minute par microgramme. Cette activité reflète l'intensité de synthèse de l'ADN dans l'intestin.

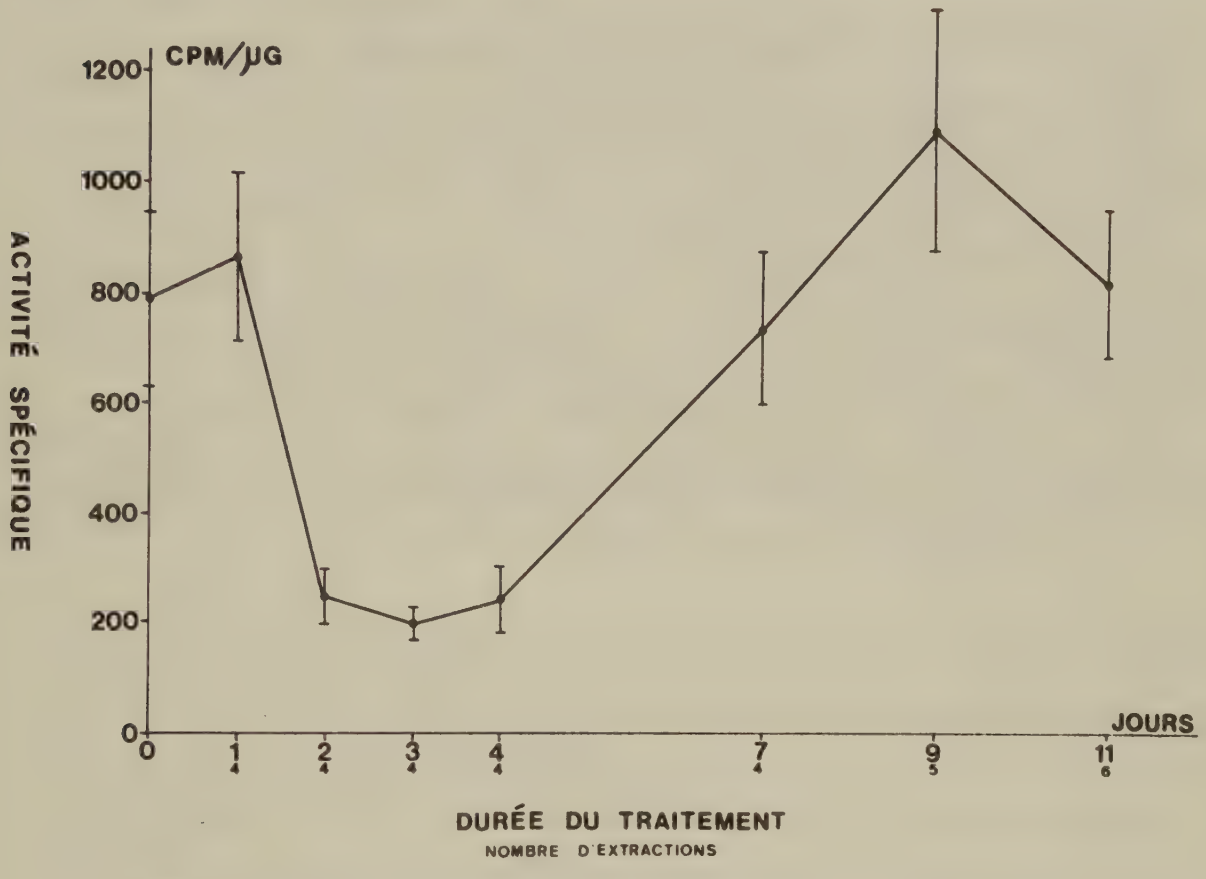
RÉSULTATS. — La valeur moyenne de l'activité spécifique de l'ADN intestinal et son erreur-standard sont indiquées sur la figure, pour chaque durée du traitement.

Une exposition de 1 jour ne modifie pratiquement pas la vitesse de synthèse de l'ADN, puisque l'activité spécifique (869 cpm/µg) demeure voisine de celle que l'on obtient chez les larves témoins en prémétamorphose (795 cpm/µg).

Si les animaux sont traités de 2 à 4 jours, la vitesse de synthèse de l'ADN diminue brutalement. L'activité spécifique chute de manière significative ($p < 0,01$ entre 1 et 2 jours), et sa valeur moyenne se situe entre 200 et 250 cpm/µg.

Du 4$^e$ au 9$^e$ jour, la vitesse de synthèse de l'ADN augmente. L'activité spécifique s'accroît significativement du 4$^e$ au 7$^e$ jour ($0,01 < p < 0,02$) et culmine au 9$^e$ jour (1 107 cpm µg).

Au-delà du 9$^e$ jour, la vitesse de synthèse de l'ADN décroît à nouveau, et l'activité spécifique s'abaisse à 824 cpm/µg au 11$^e$ jour de traitement.




Effet du traitement par la thyroxine sur l'activité spécifique de l'ADN intestinal, chez des larves d'Alyte en prémétamorphose. Les traits verticaux indiquent les erreurs-standard.

Discussion. – Les variations subies par l'activité spécifique de l'ADN s'accordent avec les observations radioautographiques conduites au niveau intestinal [(7) et (10)]. L'épithélium primaire est le seul responsable de la chute d'activité apparue entre le 1$^{er}$ et le 2$^e$ jour de traitement. L'hormone freine en effet la croissance de cette assise, où les noyaux marqués deviennent rares. L'épithélium secondaire est alors très peu développé, et la zone extra-épithéliale demeure pauvre en noyaux marqués.

Du 2$^e$ au 4$^e$ jour, des signes de dégénérescence apparaissent dans l'épithélium primaire, où l'incorporation du radioprécurseur cesse. D'autre part, en dépit d'un indice de marquage nucléaire qui augmente, le nombre des noyaux marqués reste réduit dans l'épithélium secondaire dont le volume est encore très faible. Enfin, l'indice de marquage nucléaire s'accroît à peine dans la zone extra-épithéliale. De telles observations sont compatibles avec les valeurs particulièrement basses de l'activité spécifique, lors de cette phase du traitement.

Si les larves sont exposées pendant 7 jours à l'action de la $T_4$, l'élévation de l'activité spécifique de l'ADN est principalement due à la multiplication des cellules de la zone extra-épithéliale, où les noyaux marqués deviennent fréquents.

Au 9e jour, par contre, l'épithélium secondaire représente le principal responsable de la haute valeur de l'activité spécifique, puisque son volume s'est accru de manière appréciable et que, de plus, l'indice de marquage nucléaire y atteint des valeurs particulièrement grandes, voisines de 50 %. Cet épithélium de néoformation villeux obstrue alors partiellement la lumière de l'intestin.

Au 11e jour, l'abaissement de l'activité spécifique est en rapport avec une diminution de l'incorporation de la thymidine tritiée par l'épithélium secondaire et la zone extra-épithéliale.

Au cours des métamorphoses naturelle et provoquée, les remaniements intestinaux sont analogues et, à la dégénérescence de l'épithélium primaire, succèdent une prolifération de l'épithélium secondaire et un épaississement de la zone extra-épithéliale. Les variations de l'activité spécifique de l'ADN confirment cette similitude entre les deux types de métamorphoses puisque, dans l'un et l'autre cas, l'activité diminue avant de présenter un pic ([11]). La phase d'activité spécifique minimale, allant du 2e au 4e jour d'exposition à la $T_4$, serait l'équivalent d'une prométamorphose naturelle, au cours de laquelle l'épithélium primaire cesse de s'accroître, alors que l'épithélium secondaire n'a pas commencé à proliférer ni la zone extra-épithéliale de s'épaissir. La période comprise entre le 4e et le 11e jour s'identifierait au climax naturel, durant lequel les grands remaniements intestinaux s'installent. Soulignons toutefois que l'activité spécifique est significativement plus élevée lors du climax provoqué (9e jour : 1 107 cpm/µg) que lors du climax spontané [333 cpm/µg, ([11])].

(*) Séance du 13 février 1978.

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*Laboratoire de Biologie-Vertébrés, Centre d'Orsay,*
*Université de Paris-Sud*, 91405 *Orsay*
et *Laboratoire de Sciences naturelles de l'E.N.S. de Saint-Cloud,*
2, *avenue du Palais*, 92211 *Saint-Cloud.*

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# Conjugated Effects of Thyroxine and X-rays on the Intestinal Wall of *Alytes obstetricans* Larvae (Anuran Amphibian)

Michel Dauça*,** and Jacques Hourdry**

* Laboratoire de Biologie et Biochimie du Développement Ecole Normale Supérieure, Saint-Cloud, France,

** Laboratoire de Biologie-Vertébrés Centre d'Orsay, Université Paris-Sud, Orsay, France

**Summary.** The conjoined effects of thyroxine and X-rays on the intestinal wall were studied using *Alytes obstetricans* tadpoles in premetamorphosis.

Thyroxine alone induces degeneration of the larval epithelium (primary epithelium) and its replacement by a secondary epithelium. The latter is derived from stem cells via the development of islets.

In animals submitted to irradiation only, many of these stem cells showed signs of necrosis.

In irradiated larvae treated with thyroxine, the secondary epitheliocytes were rare and never formed islets. Radioautographic observations confirmed their very low proliferation rate. Contrary to what was observed in the hormone treated larvae, cell fragments of the primary epithelium were extruded in the connective tissue, and phagocytes appear to infiltrate the epithelium.

In animals treated with thyroxine and later submitted to irradiation, islets of secondary epitheliocytes developed while some cells degenerated. There again, the phagocytes were noted in both the connective tissue and the epithelium.

**Key words:** X-rays – Thyroxine – Intestine – Amphibian larvae.

During metamorphosis, anuran amphibians undergo important changes (Frieden and Just, 1970), presumably induced by a rise in plasma thyroid hormone levels. This rise was suggested by Etkin (1968), who reproduced normal metamorphosis in thyroidectomized tadpoles dipped in graded series of thyroxine, and has recently been substantiated by radioimmunoassay (Buscaglia, 1976; Buscaglia and Bednar, 1976; Leloup and Buscaglia, 1977; Miyauchi et al., 1977; Regard et al., 1978). More precisely, at the time of metamorphosis, the intestine shortens, loses its whorl shape and acquires villi. In this respect, histological observations revealed the specific and yet opposed actions of the thyroid hormones. These hormones induce degeneration

*Send offprint requests to:* Dr. Michel Dauça, Laboratoire de Biologie-Vertébrés, Centre d'Orsay, Université Paris-Sud, 91405 - Orsay, France

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of the primary epitheliocytes, but stimulate proliferation of the secondary epitheliocytes derived from the stem cells (Hourdry and Dauça, 1977).

In growing tissues, the stem cells and the cells derived from them are critically sensitive to X-rays (Wiernik et al., 1962; Detrick et al., 1963; Patt and Quastler, 1963; Hugon and Borgers, 1966; Trier and Browning, 1966; Spreij, 1971; Carlson, 1974; Mc Curdy et al., 1974; Wertz et al., 1976; Haynie and Bryant, 1977; Sasaki and Sakka, 1978).

In this report, we analyse cytological effects of X-rays on the secondary development of the intestinal epithelium of animals treated with thyroxine. The changes that appeared in the intestinal connective tissue are also analyzed.

## Materials and Methods

65 tadpoles of *Alytes obstetricans*, at stages V–VII according to Taylor and Kollros (1946), were used.

### *Irradiation of Animals*

The larvae were anaesthetized with 5 % ether and placed under a lead plate with a 5 mm diameter window at the level of the abdomen. The latter received 3,500 rads from a Picker X-ray apparatus, using the following conditions: 60 KV, 5 mA, 400 rads per-minute.

### *Larvae Treated with Thyroxine*

The X-irradiation was associated with the hormonal treatment in 47 animals. The larvae were placed in a solution of Roche thyroxine ($10^{-7}$ g/ml) renewed every two days. With respect to the time of irradiation, two methods of treatment were used: a) 27 larvae were first irradiated, then placed for 4 to 11 days in thyroxine solution; b) 20 other larvae were first treated for 4 to 7 days with the hormone, then irradiated and thereafter treated again with thyroxine for 2 to 3 days.

### *Larvae not Treated with Thyroxine*

8 animals were irradiated. They did not undergo any hormonal treatment and remained for 4 to 11 days in pure spring water after irradiation.

### *Controls*

10 animals were not irradiated. 4 of them were left in spring water. The 6 others were placed for 3 to 12 days in a $10^{-7}$ g/ml thyroxine solution.

### *Histological Study*

Intestinal fragments were fixed for 50 min at 4° C, in 1 % osmium tetroxyde buffered with 0.15 M sodium cacodylate (pH 7.3). After brief washes in buffer, specimens were dehydrated in alcohol, dipped in propylene oxyde and embedded in Epon 812. Semi-thin sections were stained according to Ito and Winchester (1963) with a mixture of pyronine and toluidine blue. Ultra-thin sections were contrasted with uranyl acetate and lead citrate (Reynolds, 1963), and examined in a Jeol 100B microscope.

*Acid Phosphatase Activity*

The activity of acid phosphatase was visualized according to the technique of Miller and Palade (1964) modified by Hourdry (1974).

*Radioautographic Study*

In order to assess the proliferation rate of the various intestinal cell populations, the larvae were injected intraperitoneally with 24 μCi of $^3$H-thymidine (spec. act.: 16 to 24 Ci/mM, The Radiochemical Centre, Amersham). As previously described (Dauça and Hourdry, 1977), the labelling index was obtained for each intestinal layer.

## Results

### *Morphology*

#### Irradiated Animals not Treated with Thyroxine

X-rays did not induce any change in primary intestinal epitheliocytes. Nevertheless, many stem cells of the secondary epitheliocytes underwent degeneration. Semi-thin sections revealed the presence of toluidinophilic granulations in their cytoplasm (Fig. 1).

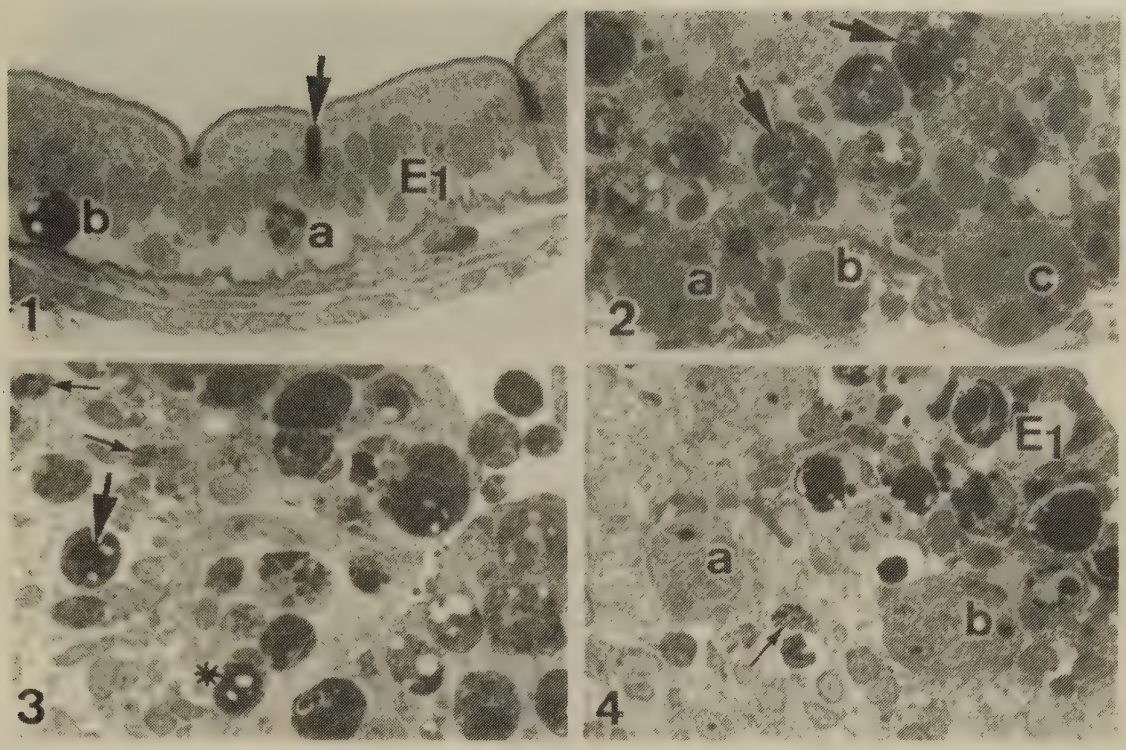
At high magnification, these granulations were identifiable as autophagic vacuoles and myeloid bodies. Some lysosomes were of considerable size, i. e. greater than 5 μm. After irradiation, nuclei of some stem cells became infolded and pyknotic.

#### Irradiated Animals Treated with Thyroxine

*Light Microscopy:* In control animals treated with thyroxine, proliferating secondary epitheliocytes formed islets below the degenerating primary epithelium (Fig. 2). In X-irradiated and hormone treated larvae, the primary epithelium degenerated. Yet, there was apparently no islet of secondary epitheliocytes (Fig. 3). Necrotized primary epitheliocytes were located close to the connective tissue which they occasionally penetrated (Fig. 3). Such an infiltration of the epitheliocytes into the connective tissue was never observed in thyroxine-treated animals. It may result from the absence of a physical barrier due to contiguous islets. Finally, phagocytes characterized by numerous toluidinophilic granulations were more numerous in the connective tissue. Some of these cells became infiltrated between primary epitheliocytes (Fig. 3).

*Electron Microscopy.* When X-irradiation was given early and followed by thyroxine treatment, electron microscopy confirmed a further series of changes in the intestinal wall:

a) Necrotized primary epitheliocytes, whether or not fragmented, were observed very close to the connective tissue (Figs. 5 and 6), or within it. These epitheliocytes contained large autophagic vacuoles and dense or myeloid residual bodies.




**Figs. 1–4.** Semi-thin sections of the intestine

**Fig. 1.** Irradiated larva maintained for 6 days in spring water. Two stem cells undergoing degeneration are localized in the base of the primary epithelium ($E_1$). One (*a*) contains large toluidinophilic granulations. The other (*b*) is completely destroyed and presents a very dense aspect. *Arrow*: mucosal secretion of the goblet mucous cell. × 450

**Fig. 2.** Non-irradiated larva treated for 8 days with thyroxine. Several cells of the primary epithelium are completely destroyed and particularly chromophilic (*arrows*). Below, 3 islets of secondary epitheliocytes are seen (*a, b, c*) × 450

**Fig. 3.** Irradiated larva treated for 9 days with thyroxine. Remnants of primary epitheliocytes are noted at the base of the epithelium (*asterisk*) and in the connective tissue (*thick arrow*). No islet of secondary epitheliocytes is visible. Several phagocytes with granular contents are noted in the connective tissue (*thin arrows*). × 450

**Fig. 4.** Larva treated for 7 days with thyroxine, irradiated, and treated again with hormone for one day. Under the primary epithelium undergoing lysis ($E_1$), 2 islets of secondary epitheliocytes (*a, b*) are observed. A phagocyte is seen in the connective tissue (*arrow*). × 590

b) No secondary epitheliocyte islets were identified with certainty. However, multiplication of these cells was suggested on some micrographs by the presence of small groups of cells with a high nuclear-cytoplasmic ratio (Fig. 6).

c) In the connective tissue, cells of various types showed pseudopodia and contained numerous dense bodies corresponding to the toluidinophilic granulations observed with the light microscope. A few of these cells contained dense bodies that were all less than 0.3 μm in size. Their nuclei were often elaborately infolded. Moreover, these observations allow us to identify these cells as neutrophilic leukocytes which have not yet participated in phagocytosis.

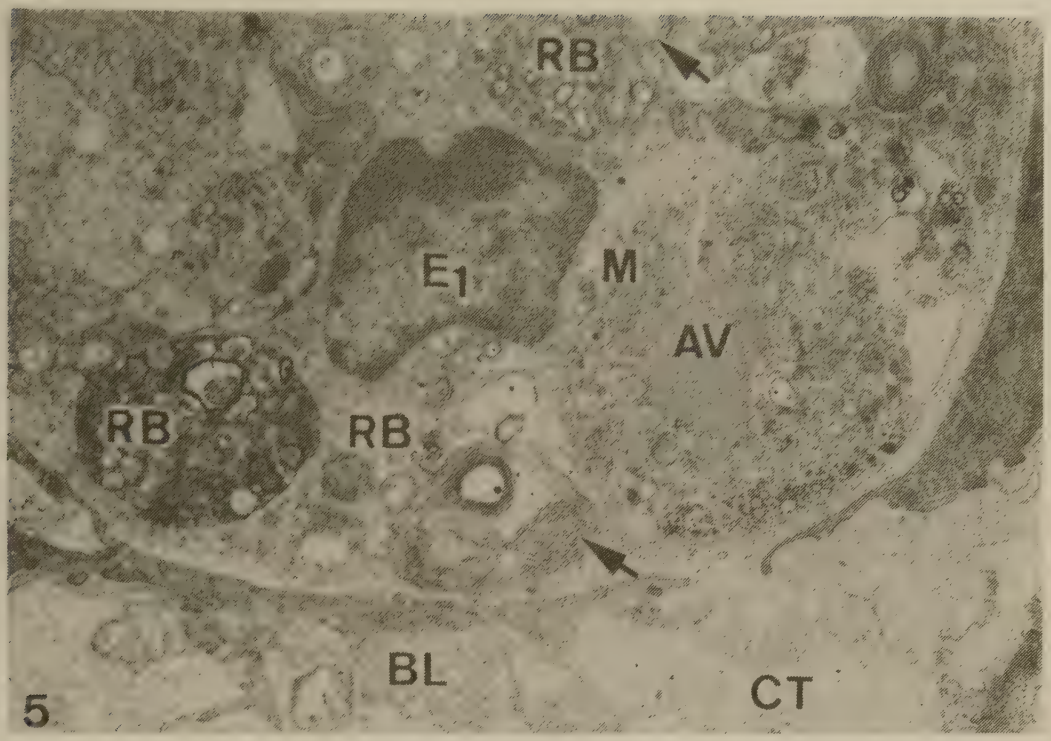



**Fig. 5.** Irradiated larva treated for 4 days with thyroxine. This primary epitheliocyte undergoing degeneration ($E_1$) comes into contact with the connective tissue (*CT*). Within one of the autophagic vacuoles (*AV*), mitochondria (*M*) are gathered together and their matrix has become clear. Several residual bodies (*RB*) have a dense content or are composed of membrane whorls (*arrows*). *BL* basal lamina. ×4500

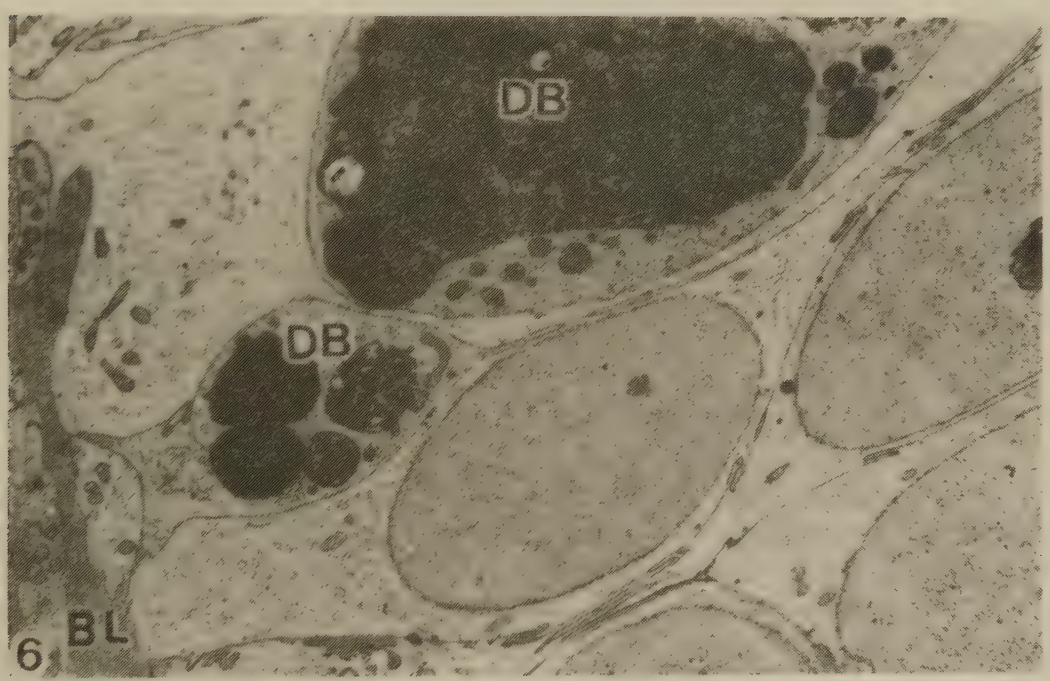



**Fig. 6.** Irradiated larva treated for 9 days with thyroxine. Near the basal lamina (*BL*), altered primary epitheliocytes contain large dense bodies (*DB*). On the right, cell proliferation is suggested by the high nuclear cytoplasmic ratio and the relatively small number of organelles. ×4100

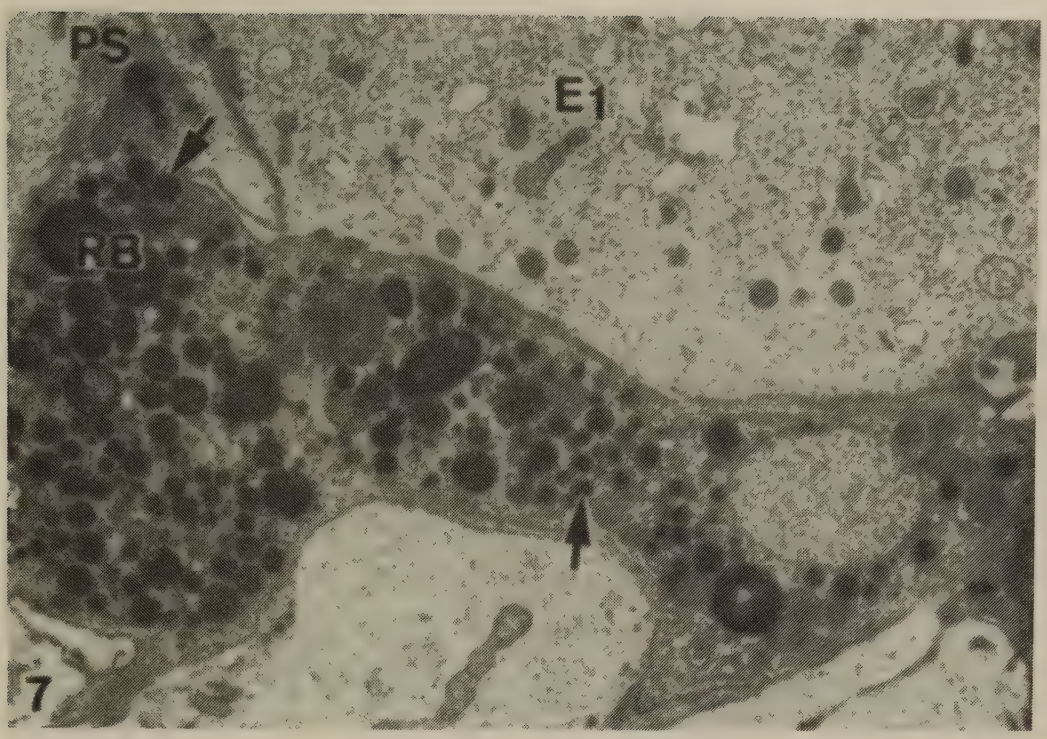


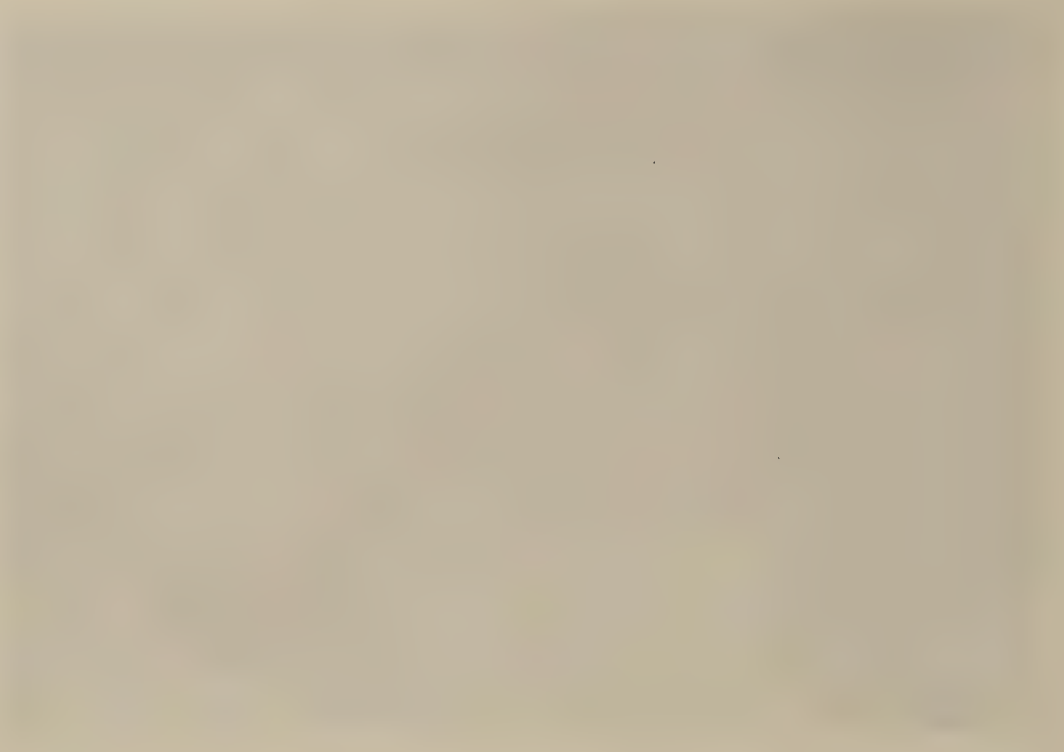

**Fig. 7.** Irradiated larva treated for 11 days with thyroxine. A phagocyte is infiltrated between the primary epitheliocytes. The cytoplasm of the phagocyte encloses two types of dense bodies. The smallest bodies would have never participated in a lytic process (*arrows*). The largest (*RB*) represent the residual forms of heterophagic vacuoles where exogenous material has been hydrolyzed. $E_1$ primary epithelium; *PS* pseudopod. ×9000

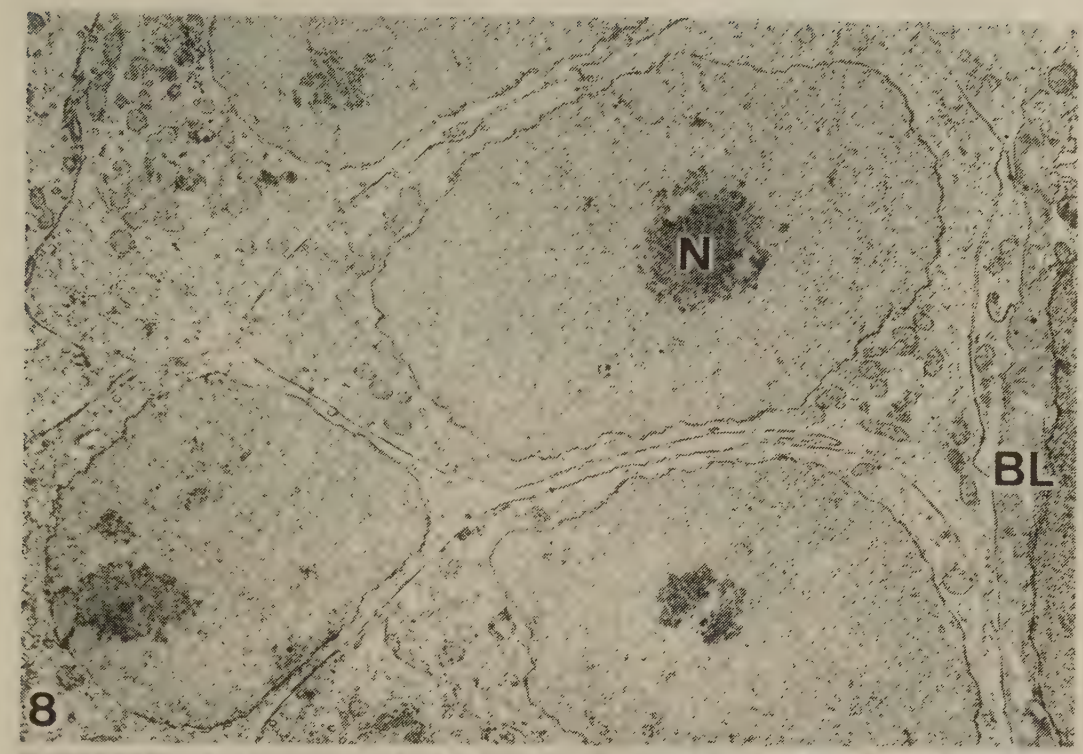
Other cells contained dense bodies of various size, some of which reaching 4 µm. These cells are probably a population of phagocytes, and their large dense bodies could represent the remnants of heterophagic vacuoles. The epithelial origin of the absorbed material is suggested by the infiltration of phagocytes between primary epitheliocytes (Fig. 7). These phagocytes may be derived from blood or have a mesenchymal origin. After irradiation, neutrophilic leukocytes and histiocytes could migrate towards the altered primary epithelium and absorb cell fragments. No phagocytosis was noted when animals were treated with thyroxine.

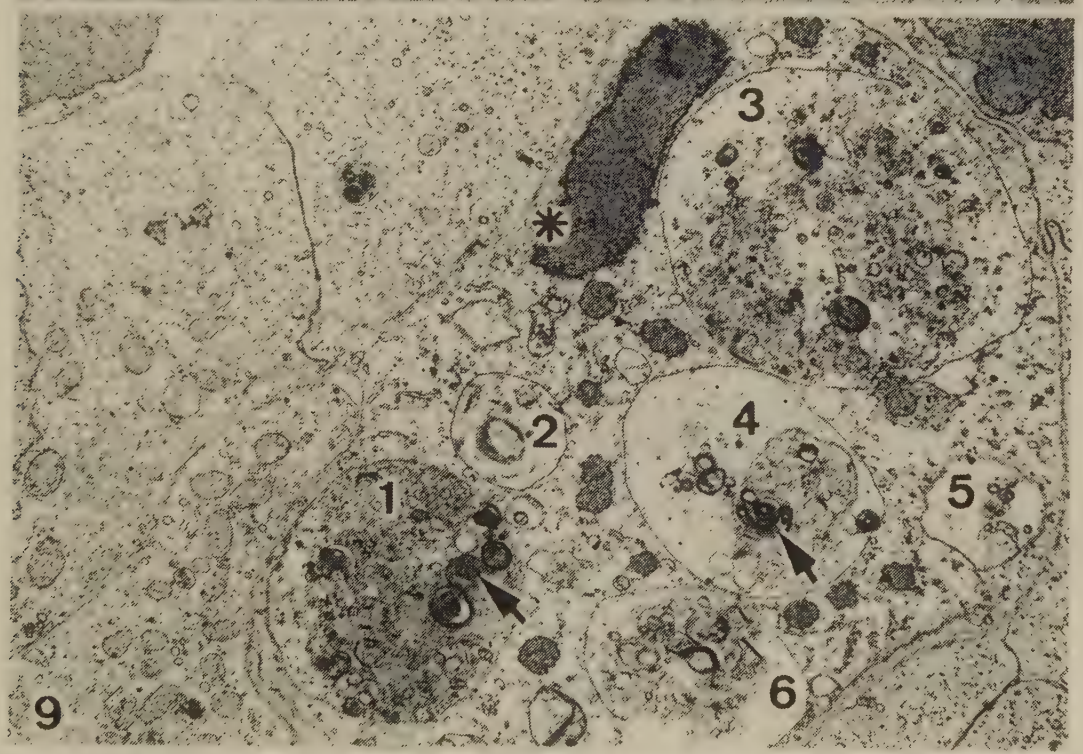
## Thyroxine-Pretreated Animals, Irradiated, and Treated Again with the Hormone

*Light Microscopy.* X-irradiation was carried out not less than 4 days after the beginning of the hormonal treatment. Small islets of secondary epitheliocytes were noted under the degenerating primary epithelium (Fig. 4). Fine toluidinophilic granulations were found in various secondary epitheliocytes. Phagocytes may be seen in the connective tissue where primary epitheliocyte fragments where rarely observed.

*Electron Microscopy.* In the islets studied, many secondary epitheliocytes appeared unchanged. Their embryonic character was indicated by a high nuclear-





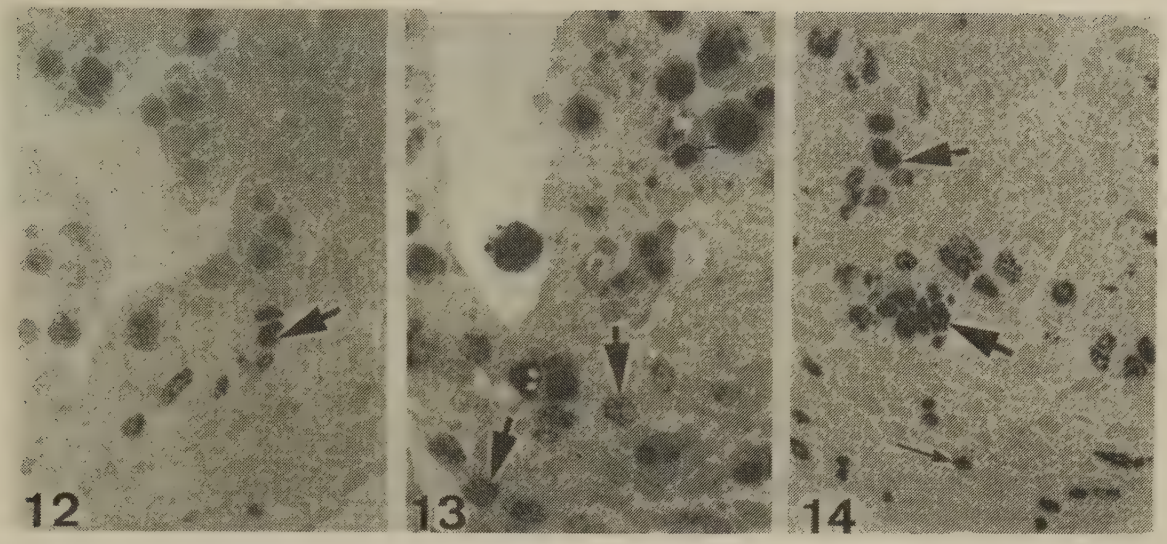

**Figs. 8 and 9.** Larvae treated for 6 days with thyroxine, irradiated, and treated again for two days with hormone

**Fig. 8.** The secondary epitheliocytes of this islet have a high nuclear-cytoplasmic ratio. Each nucleus contains a very apparent nucleolus (*N*). Areas rich in polysomes are seen in the cytoplasm. *BL* basal lamina. ×5000

**Fig. 9.** One of the secondary epitheliocytes of the islet shows signs of autolysis. Its nucleus is pyknotic and very dense to electrons (*asterisk*). Several residual bodies (*1–6*) contain a broken-up membrane material consisting of vesicles or myeloid whorls (*arrows*). ×9000

**Fig. 10.** Thyroxine-treated larva, irradiated, and treated again with the hormone. One of the secondary epitheliocytes of this islet (*3*) shows signs of degeneration. Note autophagic vacuoles and residual bodies. On the right, a phagocyte (*PC*)

**Fig. 11.** Irradiated larva not treated with thyroxine. Stem cell undergoes necrosis (*SC*) at the base of the primary epithelium ($E_1$). *Abbreviations used in Figures 10 and 11: AV* autophagic vacuole; *BL* basal lamina; *CA* capillary; *CF* collagenous fiber; *DB* dense body; *M* mitochondrion; *MW* membrane whorl; *N* nucleolus; *PS* pseudopod; *RB* residual body.




**Figs. 12–14.** Radioautograms of intestine after injection of triated thymidine to larvae: semi-thin sections

**Figs. 12 and 13.** Irradiated larva treated for 9 days with thyroxine. A few labelled nuclei are observed in the epithelium (*arrows*). They are absent in the extra-epithelial area

**Fig. 12.** No staining. × 350

**Fig. 13.** Toluidine blue staining. × 400

**Fig. 14.** Larva treated for 9 days with thyroxine. The labelled nuclei are frequent at the base of the villous secondary epithelium, which is the only present epithelium (*thick arrows*). They are also numerous in the extra-epithelial area (*thin arrow*). × 350

cytoplasmic ratio. Numerous polysomes and profiles of rough endoplasmic reticulum occurred in the cytoplasm (Fig. 8). Nevertheless, some secondary epitheliocytes showed signs of autolysis (Fig. 9). Their nucleus became pyknotic and electron dense. Autophagic vacuoles and residual bodies were observed in the cytoplasm.

Phagocytes recognizable by their pseudopodia and their dense bodies were seen in the connective tissue, and also between the degenerating primary epitheliocytes. These phagocytes may be derived from mesenchymal cells or represent a population of neutrophilic leukocytes, migrating from the capillaries.

These morphological results are summarized in Figs. 10 and 11.

## Visualization of the Acid Phosphatase Activity

This lysosomal activity was visualized on semi-thin sections. In irradiated larvae treated with thyroxine, this activity appeared in the degenerating primary epithelium. The enzymatic activity was also visualized in the epithelial remnants occurring in the connective tissue and in the lysosomes (heterophagic vacuoles and residual bodies) of the phagocytes.

**Table 1.** Determination of the labelling index (in percentages) of the intestinal epithelial and extra-epithelial zones, during the treatment of *Alytes* tadpoles by X-rays and thyroxine or thyroxine alone

| | X-rays and Thyroxine | | | | Thyroxine alone | | | |
|---|---|---|---|---|---|---|---|---|
| Duration of hormonal treatment (in days) | 3 | 4 | 7 | 9 | 3 | 4 | 7 | 9 |
| Epithelium | 0.1 | 0.1 | 1.3 | 8.2 | 1.4 | 1.2 | 5.7 | 20.6 |
| Extra-epithelial Zone | 0.1 | 0.1 | 0.4 | 1.6 | 0.3 | 1.2 | 6.0 | 6.9 |

The radioautographs were analyzed microscopically by scoring the percentage of cells with labelled nuclei (five silver grains or/more) in the epithelium and in the extra-epithelial zone (i.e: connective chorion + muscular and adventitious tissues). At the level of the epithelium, both primary and secondary (if present) epitheliocytes were considered

In non irradiated larvae treated with thyroxine, the acid phosphatase activity remained localized exclusively in the primary epithelium. It was not observed either in the secondary epitheliocyte islets or in the connective tissue, since the latter contained no phagocytes.

### Radioautographic Study

This study was carried out in thyroxine treated animals whether or not submitted to irradiation.

Examination of the intestinal radioautographs (Figs. 12–14) showed that the frequency of labelled nuclei in the epithelium and in the extra-epithelial zone, was decreased when thyroxine treatment was preceded by X-irradiation. In irradiated animals, the determination of the labelling index (Table 1) confirms the existence of a low proliferation of secondary epitheliocytes. Incorporation of $^{3}$H-thymidine into the nuclear DNA of the primary epitheliocytes, was very unlikely as these cells degenerated.

## Discussion

In growing tissues, the stem cells and the cells derived from them by proliferation are quite sensitive to X-rays as shown by the development of necrotic phenomena and the decrease of the number of mitotic figures.

In irradiated *Alytes* tadpoles, our results showed degeneration of the stem cells of the secondary intestinal epithelium. Furthermore, when secondary epitheliocytes proliferate rapidly in the presence of thyroxine, X-rays induce degeneration of some of them. Similar observations were made in cells undergoing proliferation in the intestinal crypts of irradiated mammals, where autophagic vacuoles and pyknotic nuclei were frequently described (Montagna and Wilson, 1955; Wiernik et al., 1962;

Detrick et al., 1963; Patt and Quastler, 1963; Matsuzawa and Wilson, 1965; Hugon and Borgers, 1966; Trier and Browning, 1966). Similarly, in insects, necrotic processes were induced by X-rays in imaginal wing discs of the pupa (Spreij, 1971; Haynie and Bryant, 1977) and in the epidermal stem cells (Sasaki and Sakka, 1978).

Our radioautographic observations and estimates of the labelling index showed a decrease in cell proliferation, in tadpoles submitted to irradiation and thyroxine treatment as compared with control animals treated only with the hormone. Such an X-ray effect was noted in the intestinal crypts of mammals (Wiernik et al., 1962; Hugon et al., 1965; Trier and Browning, 1966). In some urodela, a lower cell proliferation was demonstrated in the blastema of the amputated limb (Carlson, 1974; Wertz et al., 1976) and in the epidermis at the time of metamorphosis (McCurdy et al., 1974). Finally, in drosophila, the mitotic frequency was reduced in the imaginal wing disc of the pupa (Haynie and Bryant, 1977).

The low incidence of cellular divisions may be ascribed to the degeneration of undifferentiated stem cells that have been submitted to X-rays. This point of view was advanced by Sasaki and Sakka (1976, 1978) who explained the low development of the epidermis and intestinal epithelium in an irradiated fly by rarefaction of viable embryonic cells in these tissues. Such a hypothesis finds support in our observations on the intestine of irradiated *Alytes* tadpoles, since numerous stem cells of the secondary epithelium underwent degeneration.

During cell proliferation, X-rays may also interfere without inducing immediately observable changes. Thus, irradiation may prevent the initiation of the synthesis of DNA or slow down its growth (Beaupain, 1978). Such impeded elongation may be due to breaks or abnormal links ocurring in the DNA strands (Watanabe, 1974; Makino and Okada, 1975; Painter and Young, 1975; Tolmach and Jones, 1977; Han and Elkind, 1978). The amounts of DNA contained in some chromosomes may also be modified. Such modifications induced by the irradiation may lead to cell death, after several divisions (Hopwood, 1974; Han and Elkind, 1978). According to Patt and Quastler (1963), X-rays may also block the cell cycle at the end of phase $G_2$ and impede mitosis. In irradiated Alytes tadpoles treated with thyroxine, the division of the rare stem cells which are still viable in the intestinal epithelium is demonstrated by the existence of labelled nuclei belonging to secondary epitheliocytes. However, the epithelial labelling index remains low until the 7th day. This low rate of division of morphologically normal stem cells may be caused by some change in their genetic material. Similarly, in tadpoles pretreated with thyroxine, irradiated and thereafter treated again with the hormone, a change in the DNA of some secondary epitheliocytes is also possible. Such a change in the DNA would limit the mitotic possibilities of these cells.

The fact that the labelling index rises suddenly in larvae kept for 9 days in thyroxine solution after irradiation suggests that the secondary epithelium is able to recover with time. Similar observations were carried out in irradiated mammals, where the crypts may regenerate at a later stage, even after large doses of radiation. The mechanism of this recovery is still inknown.

Irradiation of *Alytes* larvae always causes the migration of phagocytes towards the intestinal epithelium. This is not seen after treatment with thyroxine alone. The origin of these cells is not clear. As already shown by Trier and Browning (1966),

some phagocytes in the human intestine might be neutrophilic leukocytes. However, the development of phagocytic activity in histiocytes, is also possible as shown in the thymus (Braun, 1965; Klug, 1965).

It appears from this study that, in an *Alytes* larva, X-rays may slow down the development of the secondary epithelium which becomes substituted for the initial epithelium, during metamorphosis induced by thyroid hormones. X-irradiation of the intestine opens new prospects in this respect. This technique will allow further studies of specialized molecules in the intestinal epithelium, such as brush border enzymes, which are qualitatively modified during metamorphosis (Dauça et al., in preparation).

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# AMPHIBIAN INTESTINAL BRUSH BORDER ENZYMES DURING THYROXINE-INDUCED METAMORPHOSIS.
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# Amphibian Intestinal Brush Border Enzymes During Thyroxine-Induced Metamorphosis*

## A Biochemical and Cytochemical Study

M. Dauça[1,2], J. Hourdry[2], J.S. Hugon[3], and D. Ménard[3,**]

[1] Laboratoire de Biologie et Biochimie du Développement, Ecole Normale Supérieure, Grille d'Honneur – Parc de Saint-Cloud, F-92211 Saint-Cloud, France,
[2] Laboratoire de Biologie-Vertébrés, Centre d'Orsay, Université Paris-Sud, F-91405 Orsay, France, and
[3] Département d'Anatomie et de Biologie Cellulaire, Unité de Biologie Intestinale, Faculté de Médecine, Université de Sherbrooke. Sherbrooke, Québec, Canada J1H 5N4

**Summary.** The development of intestinal brush border hydrolytic activities has been studied during thyroxine-induced metamorphosis of *Rana catesbeiana*. Alkaline phosphatase activity peaks at 3 and 10 days after the beginning of the thyroxine treatment. The cytochemical observations concerning alkaline phosphatase activity are in agreement with the biochemical data. At the ultrastructural level, alkaline phosphatase activity is particularly evident on the microvilli membranes of the enterocytes in the primary epithelium after 3 days and in the secondary epithelium after 10 days. γ-glutamyltranspeptidase exhibits an increase of activity between 7 and 10 days. On the other hand, glucoamylase, maltase, trehalase and leucylnapthylamidase activities decrease during thyroxine treatment, these enzymatic activities being lower than that normally observed after natural metamorphosis. The present study indicates that even though thyroxine is able to induce the morphological differentiation of the intestinal epithelium this hormone is unable to complete the enzymatic load of the new mucosa.

## Introduction

During metamorphosis, the intestine of the amphibian is drastically modified: its overall length decreases dramatically and its mucosa is completely replaced by a new epithelial layer exhibiting villi. (Carver and Frieden 1977; Griffiths 1961; Bonneville 1963; Bonneville and Weinstock 1970; Dournon and Chibon 1974; McAvoy and Dixon 1977; Dauça and Hourdry 1977, 1978). In previous works, we have analyzed the modifications of the brush border hydrolytic activities during that period. (Hourdry et al. 1979; Dauça et al. 1979, 1980a, b).

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** To whom requests for offprints should be sent

In the present study, we shall describe the alterations of the same enzymes during a thyroxine-induced metamorphosis of *Rana catesbeiana* tadpoles. Biochemical and cytochemical data are presented.

## Material and Methods

*Rana catesbeiana* tadpoles were purchased from McNally (Wisconsin, USA). Their developmental stage was determined according to Taylor and Kollros (1946). They were kept at 10° C and fed a diet supplied by McNally. Three weeks before experiments, tadpoles of stage IX-XII were carried to room temperature and treated with 0.04% thiourea in their swimming pool. Thiourea suppresses the release of endogenous thyroid hormones and thus prevents the onset of spontaneous metamorphosis. These animals are used as controls. Other tadpoles were treated with thiourea (0.04%) and DL-thyroxine (Sigma) at a final concentration of $10^{-7}$ g/ml.

*Cytochemistry.* The alkaline phosphatase localization in the control and treated animals was studied along the intestinal tract at the light and electron microscopic level after incubation in the Hugon and Borgers medium (1966) as previously described (Hourdry and Hugon 1979).

*Biochemistry.* For the biochemical study, the intestines were quickly removed and treated according to the technique already described (Dauça et al. 1979). Trehalase and maltase activities were determined according to a modification by Lloyd and Whelan (1969) of the Dahlqvist method (1964). Glucoamylase was measured using soluble starch as substrate according to Schlegel-Haueter et al. (1972). Alkaline phosphatase and γ-glutamyl-transpeptidase were measured according to Eichholz (1967) and Naftalin et al. (1969), respectively. Leucylnaphthylamidase was assayed according to the method of Goldbarg and Rutenburg (1958). Proteins were measured according to Lowry et al. (1951) and DNA according to the modifications of Burton's method (1956) by Giles and Myers (1965).

## Results

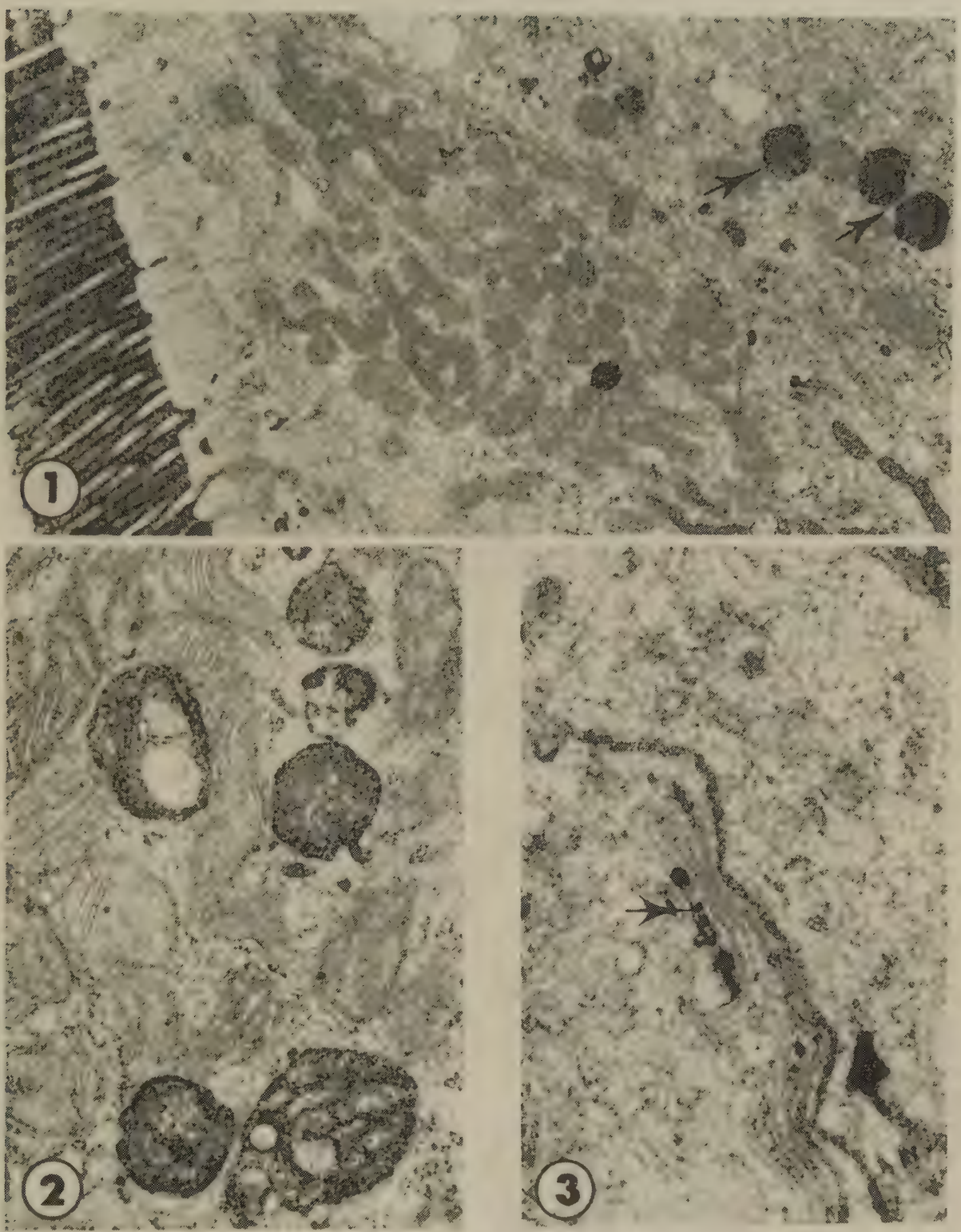
### *1) Cytochemistry*

#### a) Light Microscopy

Controls exhibit a strong reaction on the brush border of the intestinal cells and on several small vesicles in the apical part of these cells. After three days of thyroxine treatment, the same localization is observed. After seven days of thyroxine treatment, a weak positivity is noted on the degenerative epithelial cells. After ten days of treatment, lead sulfide deposits are observed only on the brush border of the new mucosal cells.

#### b) Electron Microscopy

*Controls.* The localization of the reaction product in the epithelial cells of the premetamorphosis tadpoles has been described in a previous paper. Microvilli, apical small vesicles, large membranous vacuoles and several Golgi cisternae are stained (Figs. 1–3).

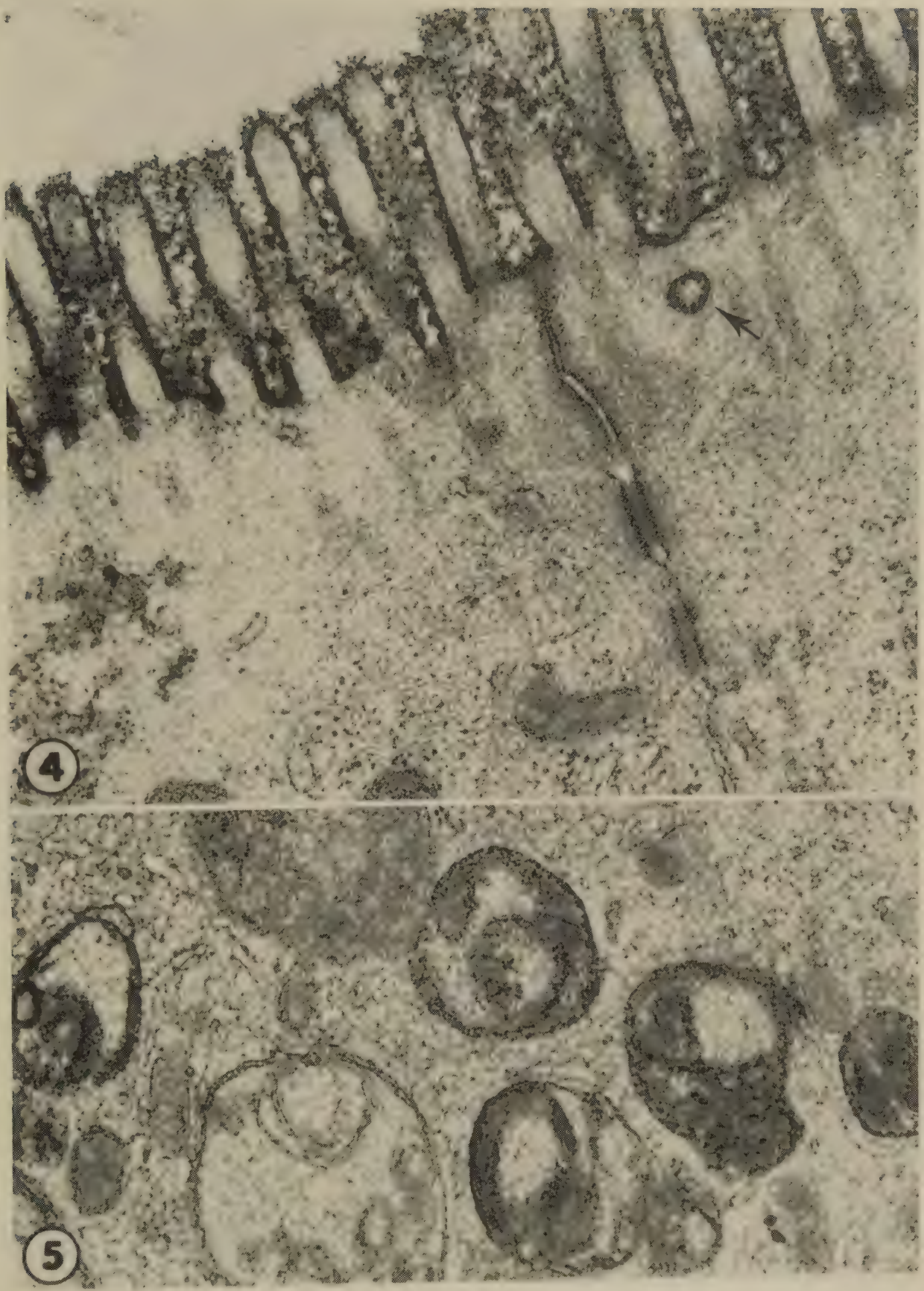



**Figs. 1–3.** Controls: ultrastructural localization of alkaline phosphatase activity. Incubation time: 90 min

**Fig. 1.** Apical part of a primary epitheliocyte. The lead phosphate precipitate is localized on the microvilli. On the right, three dense bodies are heavily marked (arrows). × 12,350

**Fig. 2.** Central part of a primary epitheliocyte. Several bodies are strongly marked. × 33,345

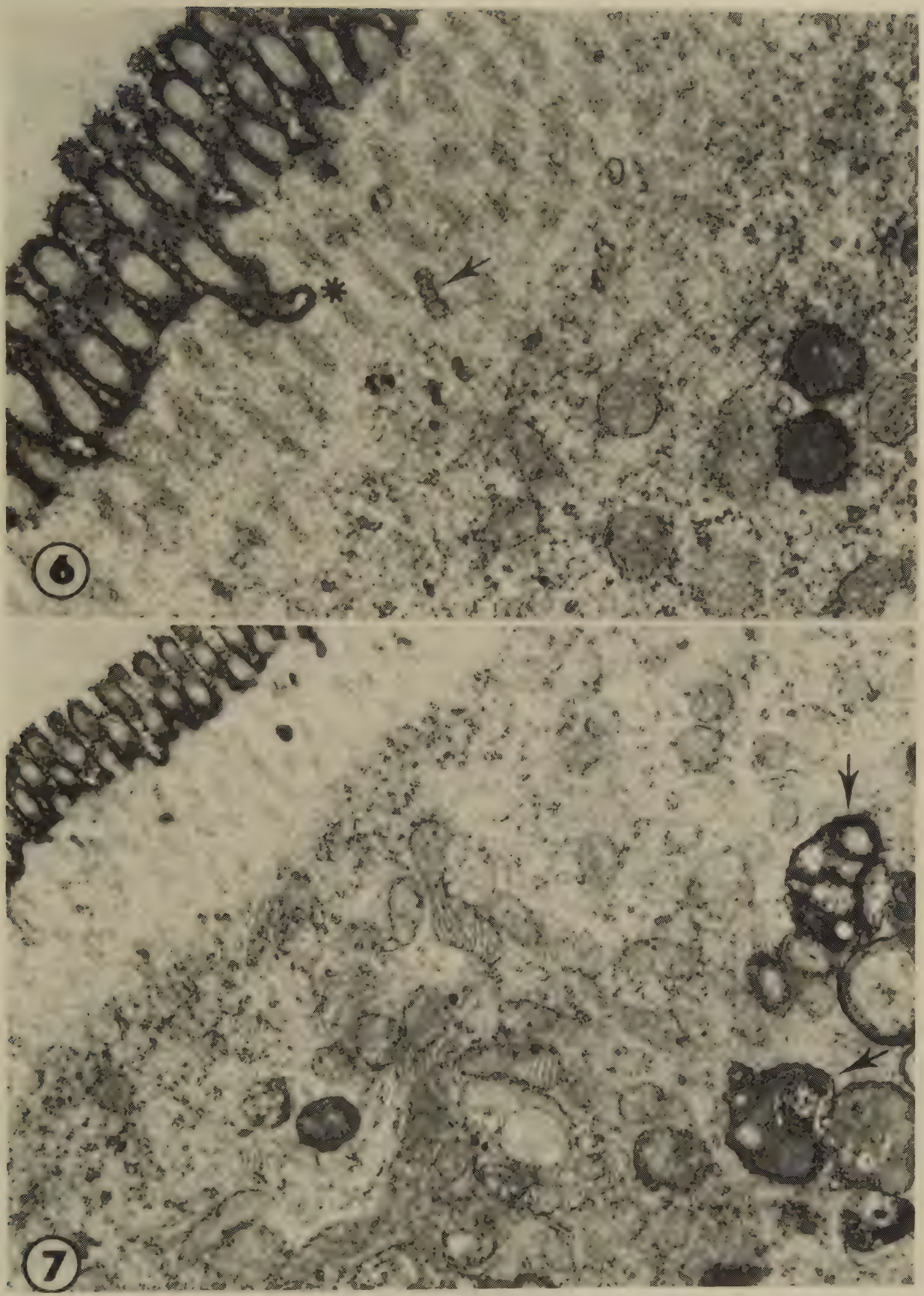
**Fig. 3.** Golgi zone of a primary epitheliocyte. This Golgi apparatus exhibits one reactive cisternae (arrow). × 63,650




**Figs. 4 and 5.** Tadpoles treated by thyroxine (3 days): ultrastructural localization of alkaline phosphatase activity. Incubation time: 90 min

**Fig. 4.** Apical part of a primary epitheliocyte. The reaction product is present on the microvilli and in a small apical vesicle located in the terminal web (arrow). ×46,170

**Fig. 5.** Central part of a primary epitheliocyte. Several bodies are filled with the reaction product. ×46,170




**Figs. 6 and 7.** Tadpoles treated by thyroxine (15 days): ultrastructural localization of alkaline phosphatase activity. Incubation time: 90 min

**Fig. 6.** Apical part of a secondary epitheliocyte. The lead phosphate precipitate is seen on the microvillus membrane, in an invagination of the apical membrane * and in an apical vesicle (arrow). The reaction product is also present in two dense bodies. ×29,850

**Fig. 7.** Apical part of a secondary epitheliocyte. The reaction product is located on the microvilli and in several heterogeneous bodies (arrows). ×16,790

**Table 1.** Intestinal enzyme activities during $T_4$-induced metamorphosis

| | Thyreostatic tadpoles (Controls) | Days after DL-Thyroxine treatment | | | |
|---|---|---|---|---|---|
| | | 3 | 7 | 10 | 15 |
| Alkaline phosphatase | 22.19 ± 2.96[a] (5) | 38.24 ± 5.10 (4) | 18.25 ± 3.78 (4) | 28.83 ± 4.03 (4) | 19.60 ± 1.43 (6) |
| γ-Glutamyl transpeptidase | 29.78 ± 3.27[b] (5) | 30.57 ± 7.70 (4)[c] | 52.35 ± 4.94 (4) | 52.15 ± 8.25 (4) | 30.93 ± 4.41 (7) |
| Glucoamylase | 12.66 ± 1.72 (5) | 12.53 ± 1.37 (4) | 7.16 ± 0.44 (4) | 5.79 ± 0.66 (4) | 4.50 ± 0.21 (7) |
| Maltase | 30.35 ± 1.90 (5) | 24.97 ± 2.78 (4) | 22.13 ± 1.22 (4) | 23.05 ± 3.84 (4) | 15.26 ± 1.01 (7) |
| Trehalase | 2.86 ± 0.54 (5) | 1.75 ± 0.27 (4) | 0.83 ± 0.42 (4) | 0.44 ± 0.01 (4) | 1.22 ± 0.27 (7) |
| Leucyl-Naphthylamidase | 23.07 ± 6.72 (5) | 10.93 ± 1.77 (4) | 7.64 ± 1.00 (4) | 6.50 ± 2.04 (4) | 8.40 ± 1.16 (8) |

[a] Specific activity in I.U/g protein
[b] Mean ± SEM
[c] Number of independant determinations. One thyreostatic tadpole and 2–3 treated larvae were respectively used for each control or experimental determination

*Treated Animals.* After three days of thyroxine treatment, the localization and the intensity of the reaction are identical to controls (Figs. 4–5). After seven days, the staining is faint and irregular on the degenerative primary epithelial cells. After 10 days, lead phosphatase deposits are again present on the microvilli, apical vesicles or vacuoles and in the Golgi stacks (Figs. 6–7).

### *2) Biochemistry*

Table 1 summarizes the behaviour of the bruh border hydrolytic activities during the thyroxine-induced metamorphosis. The activities of glucoamylase, maltase, trehalase and leucylnaphthylamidase decrease significantly during the first ten days of treatment. At day 15, only trehalase and leucylnapththylamidase activities have increased, these activities representing, however only half of those of the control animals. Alkaline phosphatase activity increases after three days of treatment, decreases at day 7 and reaches a maximum at day 10. On the other hand, γ-glutamyltranspeptidase exhibits maximal activity between day 7 and day 10.

The same patterns are observed when the enzymatic activities are compared to the DNA content instead of the protein content.

## Discussion

The morphological modifications occurring at the intestinal level during metamorphosis of amphibian are well documented (Hourdry and Dauça 1977). Thy-

roxine treatment of anuran larvae induces the same histological modifications and it is generally accepted that these changes occurring during normal metamorphosis are under the influences of thyroid hormones. Indeed, the circulating level of thyroid hormones reaches its maximum at the climax of metamorphosis (Etkin 1968; Just 1972; Leloup and Buscaglia 1977; Miyauchi et al. 1977; Regard et al. 1978; Mondou and Kaltenbach 1979).

These morphological changes are associated with alterations of the brush border membrane hydrolytic activities (Dauça et al. 1979, 1980a, b). In order to verify whether thyroid hormones were also responsible for the functional maturation of the brush border membrane hydrolytic activities, metamorphosis was induced by thyroxine. The present study shows that the activity of the brush border enzymes are significantly lower when the metamorphosis is induced experimentally by thyroxine as compared to the activities observed after natural metamorphosis (Hourdry et al. 1979). Indeed the activity of glucoamylase, maltase, trehalase and leucylnaphthylamidase does not increase during thyroxine-induced metamorphosis as already reported for natural metamorphosis (Hourdry et al. 1979; Dauça et al. 1979, 1980a) but instead decreases (Table 1) Alkaline phosphatase as well as $\gamma$-glutamyltranspeptidase exhibits a different pattern. During the thyroxine-induced metamorphosis, alkaline phosphatase activity peaks at three and ten days. These biochemical data correlate well with the present cytochemical observations (Figs 1–7). Furthermore the ultrastructural localization of alkaline phosphatase during induced-metamorphosis is in agreement with that reported during normal metamorphosis of *Rana catesbeiana* (Hourdry and Hugon 1979) as well as *Bufo vulgaris* (Chieffi and Carfagna 1960), *Rana temporaria* (Brown and Millington 1968) and *Rana pipiens* (Kaltenbach et al. 1977). In *Rana pipiens*, Kaltenbach et al. (1977) reported that thyroxine treatment also induced an increase of alkaline phosphatase activity. It is interesting to note that alkaline phosphatase activity presents a maturation process during the development of *Rana catesbeiana*, its relative mobility being always faster in tadpoles as compared to young and adult bullfrogs after gel electrophoretic separation of SDS solubilized membranes (Dauça et al. 1980b).

$\gamma$-glutamyltranspeptidase activity increases during thyroxine treatment and exhibits a maximum activity between 7 to 10 days, at which time the histolytic and histogenic processes being at their maximum level. The precise physiological role of intestinal brush border $\gamma$-GT remains undefined but the involvement of this enzyme in amino acids and peptides absorption has been put forward (Garvey et al. 1976; Meister et al. 1977). An increased $\gamma$-GT activity during a period when the animals are fasting could suggest a role in the digestion and/or absorption of metabolites allowing or facilitating the histogenesis of the new mucosa. However, this interpretation remains hypothetical as the precise localization of $\gamma$-GT during this period as well as its physiological role remain to be defined.

The present data are at variance with the behaviour of the same enzymes during natural metamorphosis and suggest that thyroxine is not the sole hormone involved in the functional maturation of the brush border membranes even though it induces all the morphological modifications normally seen during metamorphosis. Adrenal glands become functional at the metamor-

phosis (Stenger and Charipper 1946; Dale 1962; Rapola 1963). Glucocorticoid hormones would appear a possible candidate in the regulatory mechanism of the development of the brush border membrane functions. Indeed, it has been shown that glucocorticoid hormones increase alkaline phosphatase activity in tadpole intestine (Verne and Hébert 1949; Chieffi and Carfagna 1960). In salmon, it has been reported that the modifications of intestinal tract occurring during the migration are concomitant to the increase of circulating corticosteroid hormones (Hane and Robertson 1959; Robertson and Wexler 1960). In birds, thyroxine and glucocorticoid hormones are responsible for the enzymatic maturation of the small intestine (Moog and Richardson 1955; Moog 1961). In mammals, morphological as well as enzymatic modifications occurring at the intestinal level during weaning are regulated by glucocorticoids and thyroxine (Moog 1971; Moog et al. 1973; Yeh and Moog 1977; Koldovsky 1972; Malo and Ménard 1979, 1980).

The present observations indicate that even though thyroxine is able to provoke the morphological differentiation of the intestinal epithelium in thyroxine-induced metamorphosis, this hormone is unable to complete the enzymatic load of the new mucosa.

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# AMPHIBIAN INTESTINAL BRUSH BORDER MEMBRANES—I. ISOLATION FROM *RANA CATESBEIANA* TADPOLE

M. DAUCA[1,2], J. HOURDRY[2], J. S. HUGON[3] and D. MENARD[3,*]

[1] Laboratoire de Biologie et Biochimie du Développement, Ecole Normale Supérieure, 2, Avenue du Palais, 92211—Saint-Cloud, France,
[2] Laboratoire de Biologie-Vertébrés, Centre d'Orsay, Université Paris-sud, 91405—Orsay, France and
[3] Département d'Anatomie et de Biologie Cellulaire, Unité de Biologie Intestinale, Faculté de Médecine, Université de Sherbrooke, Sherbrooke, Québec, Canada J1H 5N4

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**Abstract**—1. Intestinal brush border membrane vesicles have been isolated form *Rana catesbeiana* tadpole.

2. Electron microscopy of brush border membrane vesicles demonstrates a fairly homogenous preparation of vesicles, some of them still containing electron dense material.

3. The dense vesicles probably comprise both microvillus core and membrane.

4. Negative staining of vesicles reveals the presence of knob-like structures (particles) covering the outer surface of the membrane.

5. The membranous fraction is characterized by a high specific activity of alkaline phosphatase, trehalase, glucoamylase, maltase and γ-glutamyltranspeptidase.

## INTRODUCTION

Intestinal brush border membranes have been extensively studied in the past years in a variety of mammals including man. Unfortunately, no study has been done concerning the brush border membrane of amphibian small intestine. This is surprising as the small intestine of anuran amphibian represents an interesting model. Indeed, the gut undergoes important alterations during metamorphosis, the intestinal primary epithelium being replaced by a new mucosa showing villi (Hourdry & Dauça, 1977).

Recently, Hourdry *et al.* (1979) have studied, in developing *Rana catesbeiana*, the activities of different hydrolases known to be localized in the intestinal brush border membrane in mammals (Crane *et al.*, 1976). These authors have noted the presence of the activities of alkaline phosphatase, γ-glutamyltranspeptidase, glucoamylase, maltase and trehalase in intestinal homogenates. They have also noted the absence of sucrase and lactase activities. Interestingly, all the activities présent were found to be increased in newly metamorphosed animals.

The present paper reports the successful isolation of the intestinal brush border membranes from *Rana catesbeiana* tadpoles and confirms the membrane-bound status of the different hydrolase activities found previously in homogenates.

## MATERIAL AND METHODS

### *Animals*

*Rana catesbeiana* tadpoles were purchased from Mogul, WI, U.S.A., kept in well water at 10°C and fed dry diet supplied by Mogul. One week before the experiments started, the tadpoles were put in an aquarium at room temperature. The animals used for the experiments weighed between 7–9 g and were at stage I–VII according to Taylor & Kollros (1946).

### *Brush border membrane isolation*

The original human brush border membrane isolation procedure of Schmitz *et al.* (1973) was applied to tadpole intestinal mucosa. The whole small intestine was removed, split open, washed in ice-cold saline and the mucosa was scraped off. A 1% homogenate was made in 50 mM mammitol–2 mM Tris (pH 7.1) at 4°C, using an Omnimixer (Sorvall) equipped with a microattachment, at full speed (2000 rev/min) for four periods of 15 sec followed by a continuous homogenization of 60 sec. Solid $CaCl_2$ was added to a final concentration of 10 mmol/l. Centrifugation of the calcium-treated homogenate at 2000 *g* for 10 min yielded a pellet ($P_1$) which was resuspended in 50 mM mammitol–2 mM Tris for further analysis, and a supernatant ($S_1$). The supernatant was centrifuged at 20,000 *g* for 15 min to yield a small pellet ($P_2$) containing the brush border fragments and a final supernatant ($S_2$). $P_2$ was resuspended in ice-cold redistilled water.

### *Assay methods*

Trehalase and maltase activities were assayed according to a modification by Lloyd & Whelan (1969) of Dahlqvist's method (1964). Glucoamylase was measured using soluble starch as substrate according to Schlegel-Haueter *et al.* (1972). Alkaline phosphatase and γ-glutamyltranspeptidase were measured according to Eichholz (1967) and Naftalin *et al.* (1969), respectively. Proteins were assayed according to Lowry *et al.* (1951).

### *Electron microscopy*

$P_2$ was fixed according to Graham & Karnovsky (1966) with slight modifications. Brush border fragments ($P_2$) were fixed in a mixture of 1% glutaraldehyde and 1.5% paraformaldehyde (final concentration) buffered with 0.1 M

* To whom requests for reprints should be sent.

cacodylate (pH 7.2) for 60 min at room temperature. The fixed pellet was washed in sodium cacodylate buffer and further fixed in 1% osmium tetroxide in cacodylate buffer containing 0.9 mM $CaCl_2$ for 1 hr at room temperature in the dark. Then, the fixed pellet was washed in cacodylate buffer, dehydrated in alcohol and stained for 1 hr in 0.5% uranyl acetate. After two changes in propylene oxide, the pellet was cut into small pieces and embedded in Epon. Ultra-thin sections were stained with lead citrate (1963) and examined in a Philips 300 electron microscope.

Freshly prepared 2% phosphotungstic acid adjusted to pH 7.2 with 1.0 N NaOH, was used for negative staining. A formvar-coated copper grid was allowed to float on a drop of the brush border membrane suspension for 1 min and excess fluid was removed with filter papers. The grid was then brought into contact with a drop of 2% phosphotungstic acid solution. After 15 sec the grid was taken out, the excess fluid removed with filter papers and immediately examined in a Philips 300 electron microscope.

## RESULTS

Even though the amount of material obtained in $P_2$ is relatively low when this fraction is isolated from a single tadpole, all the experiments were performed on single animals.

All data is summarized in Table 1. The purification is estimated by the rise in specific activities of microvillous enzymatic markers. In fraction $P_2$, 2% of the total proteins are found. Alkaline phosphatase activity shows an increase of 13 times in the $P_2$ fraction

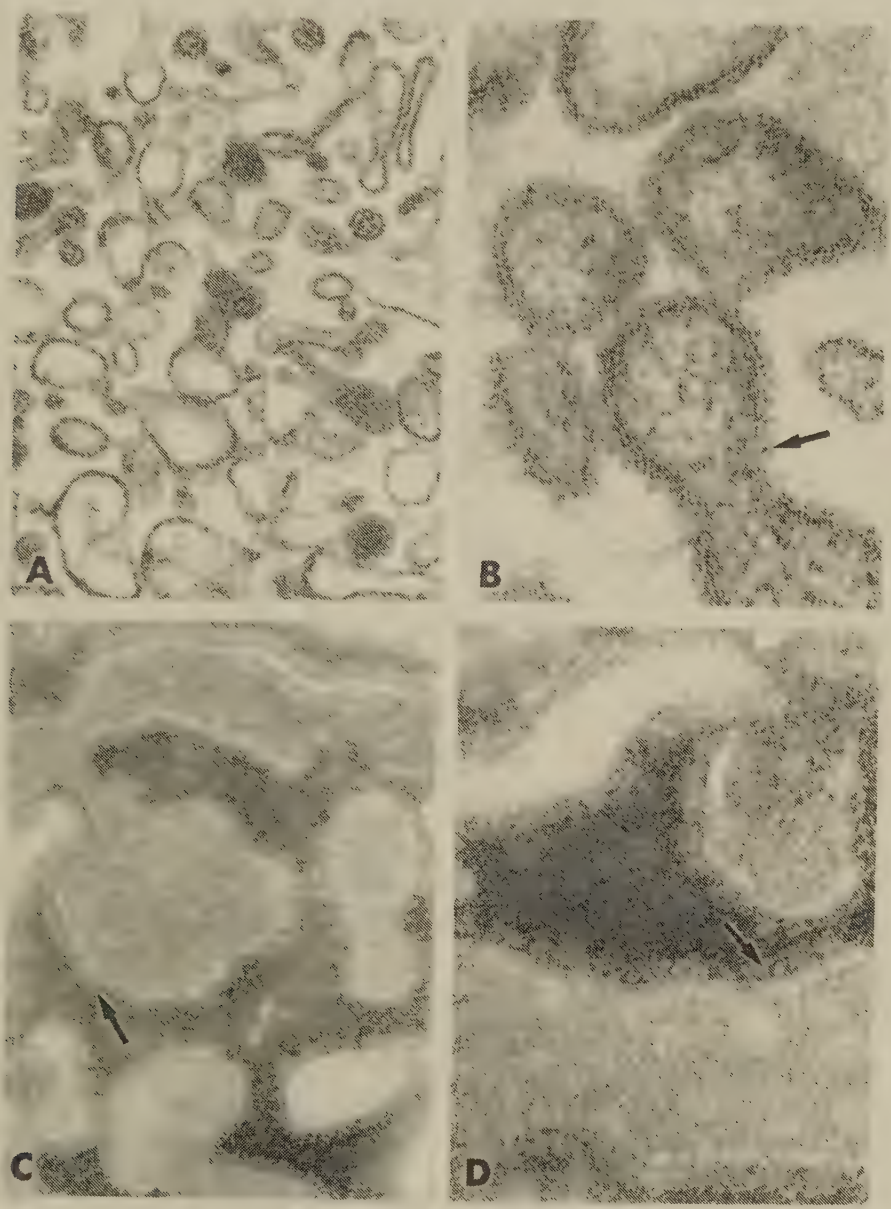


Fig. 1. Electron microscopic study of the microvillus membranes. (Fraction $P_2$).

A. This micrograph is the average representative of several micrographs which were obtained from 10 grids containing sections prepared from five different blocks. Fraction $P_2$ is essentially composed of membranous fragments forming vesicules or tubules of various shapes and sizes. Some vesicules still contain electron dense material. These dense vesicules probably comprise both microvillus core and membrane. Organelles other than electron dense vesicules could not be identified. × 50,000.

B. At higher magnification, the trilaminar structure of the membrane is clearly seen as well as the fuzzy coat (arrow). × 165,000.

C–D. Electron micrographs of negatively stained microvillus membranes. The outer surface of the membrane is covered with knob-like structures (arrows). C: × 56,000; D: × 100,000.

Table 1. Enzymatic composition of microvillus membranes

| Fraction | Protein % | Alkaline phosphatase Sp.act.* | Alkaline phosphatase % | γ-Glutamyltranspeptidase Sp.act* | γ-Glutamyltranspeptidase % | Glucoamylase Sp.act.* | Glucoamylase % | Maltase Sp.act.* | Maltase % | Trehalase Sp.act.* | Trehalase % |
|---|---|---|---|---|---|---|---|---|---|---|---|
| H | 100 | 173 ± 14† (32)‡ | 100 | 7.8 ± 0.7 (13) | 100 | 43 ± 3 (12) | 100 | 60 ± 6 (11) | 100 | 7.6 ± 0.8 (11) | 100 |
| $P_1$ | 47.5 ± 1.4 (32) | 165 ± 14 (32) | 47.6 ± 1.5 | 10.2 ± 0.9 (13) | 69 ± 3 | 37 ± 4 (12) | 43.2 ± 1.6 | 56 ± 6 (11) | 45.5 ± 2.7 | 7.3 ± 0.8 (11) | 49.2 ± 2 |
| $P_2$ | 2 ± 0.1 (32) | 2131 ± 177 (32) | 14.3 ± 0.9 | 73 ± 10 (13) | 17.8 ± 1 | 423 ± 37 (12) | 19 ± 1.4 | 572 ± 59 (11) | 18.5 ± 1.8 | 72 ± 11 (11) | 17.8 ± 1.2 |
| $S_2$ | 25.7 ± 1 (32) | 50 ± 6 (32) | 8.6 ± 1.2 | 2 ± 0.4 (13) | 5.7 ± 0.6 | 22 ± 3 (12) | 15.5 ± 2.2 | 41 ± 6 (11) | 18.3 ± 1.8 | 1.9 ± 0.3 (11) | 7.5 ± 1.5 |
| Recovery§ | 75.3 + 1.9 | | 70 ± 2.3 | | 92.7 ± 2.8 | | 77.6 ± 2.2 | | 82.3 ± 3.3 | | 74.5 ± 2 |

* Sp.act. = Specific activity in IU/g protein.
† Mean ± SE
‡ Number of different tadpoles studied.
§ Recoveries are means of the recoveries.

in comparison with the specific activity present in the homogenate (H). Trehalase, maltase, glucoamylase and γ-glutamyltranspeptidase activities are purified to a similar extent (about 9–10 times) but somewhat less than alkaline phosphatase. Depending on the enzymatic activity, the recoveries range between 70–93%. This variation could be explained by the difficulty to resuspend $P_1$, leading to an underestimation of the enzymatic activities in this fraction. It is worth pointing out that 14–19% of the total original enzymatic activity is retained in fraction $P_2$ for all enzymes.

Electron microscopy of fraction $P_2$ reveals a fairly homogenous preparation of membranous vesicules, although the vesicules are often still filled with electron dense material which presumably originated from the core of the microvilli (Fig. 1A). At high magnification (Fig. 1B), the fine structure of the membrane is clearly seen. The outer surface of the membrane has a granular appearance. Organelles other than clear and electron dense vesicules could not be identified.

Electron micrographs of negatively stained brush border membranes reveal the presence of repeating knob-like structures on the outer surface of the membranes (Figs 1C and 1D). The inner membrane surface is free of any material. These knob-like structures (particules) are approximately 40–60 Å in diameter.

## DISCUSSION

The present study shows that the $Ca^{2+}$ precipitation procedure, originally developed by Schmitz *et al.* (1973) for the isolation of human intestinal brush border membranes can be applied with success to amphibian intestine. Furthermore, it appears that the enzymatic activities previously measured on intestinal homogenates in tadpoles and adult *Rana catesbeiana* (Hourdry *et al.*, 1979) are indeed intestinal brush border enzymes, as in mammals (Crane *et al.*, 1976).

The mean increase in alkaline phosphatase specific activity of 13-fold over the homogenate compares well with the 14-fold increase in this brush border marker obtained by Schmitz *et al.* (1973) in human small intestine. Furthermore, Raul *et al.* (1978) report an identical purification factor for alkaline phosphatase in adult rat. Kessler *et al.* (1978), using a slight modification of the original procedure, observe a 15-fold purification in fraction $P_2$ over the homogenate in rat, hamster and rabbit small intestine. One has to remember that even though alkaline phosphatase is mainly localized in the brush border membrane, this enzymatic activity can be detected in lysosomes and in the Golgi region (Chieffi & Carfagna, 1959; Brown & Millington, 1968; Kaltenbach *et al.*, 1978). Trehalase, glucoamylase, maltase and γ-glutamyltranspeptidase copurified at the same extent i.e. 10-fold increase. Again, these purification factors generally agree with that reported in the literature for various mammals. For example, Galand & Forstner (1974) reported a 10-fold increase for maltase and a 13-fold increase for trehalase in suckling rats while a 12-fold increase for maltase was achieved by Koldovsky (1974). In man, a 14-fold increase of trehalase activity over the homogenate was recorded (Schmitz *et al.*, 1973). The application of the procedure developed for human small intestine to amphibian small intestine has led us to the isolation of membranous fraction ($P_2$) as pure as that reported in the literature. It is worth to point out that 15–19% of the enzymatic activities measured in the homogenate are recovered in the membranous fraction isolated form *Rana catesbeiana* tadpole as compared as more than 25% in mammals (Schmitz *et al.*, 1973; Kessler *et al.*, 1978; Forstner *et al.*, 1968; Starita Geribaldi *et al.*, 1977). This difference in the yields could be explained by a greater loss in fraction $P_1$ observed in this study.

Amphibian intestinal brush border membranes observed at the ultrastructural level are similar to that obtained from chick intestine (Tilney & Moosecker, 1971) and from mammals (Schmitz *et al.*, 1973; Forstner *et al.*, 1968; Hubscher *et al.*, 1965; Billington & Nayudu, 1975; Seetharam *et al.*, 1977). Indeed, the microvillous membranes are present as vesicules, the outer surface of the membrane exhibiting a granulated coat. Negative staining reveals the presence of knob-like structures covering the outer surface of the membrane (Figs 1C–D). These particules seem to be identical to that reported in rat (Louvard *et al.*, 1973; Louvard *et al.*, 1975), hamster (Johnson, 1969; Benson *et al.*, 1971), rabbit (Louvard *et al.*, 1975; Oda & Seki, 1965), hog (Louvard *et al.*, 1975; Maroux & Louvard, 1977) and man (Maestracci, 1976). These particules would be specific to intestinal brush border membranes as they are absent in kidney brush border membranes (Berger & Sacktor, 1970). This observation in amphibian is interesting and would suggest the existence of a remarkable structural unity among vertebrates. We could foresight a relationship between these particules and the enzymatic activities associated with the membrane similar to that established in mammals (Louvard *et al.*, 1975; Benson *et al.*, 1971; Maestracci, 1976; Oda & Seki, 1966). The use of intestinal brush border membrane vesicles is an important tool to study the mechanisms of transport. Recently, it has been shown that fraction $P_2$ can be used as is or with slight modifications for such studies (Kessler *et al.*, 1978; Lemaire & Maestracci, 1978). In the past years, small intestine of amphibian has been used to study the mechanisms of absorption of sugars (Parsons & Prichard, 1965, 1971) and there is no doubt that the possibility to isolate membrane vesicles from amphibian intestine could contribute significantly to our knowledge of the intestinal physiology. Furthermore, this work brings out the possibility to compare and to characterize the composition of amphibian intestinal brush border membranes. This aspect is presently under investigation.

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AMPHIBIAN INTESTINAL BRUSH BORDER MEMBRANES.

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# AMPHIBIAN INTESTINAL BRUSH BORDER MEMBRANES—II. ISOLATION FROM *RANA CATESBEIANA* ADULT

M. Dauca[1,2], J. Hourdry[2], J. S. Hugon[3] and D. Menard[3]*

[1]Laboratoire de Biologie et Biochimie du Développement, Ecole Normale Supérieure, 2, Avenue du Palais, 92211-Saint-Cloud,
[2]Laboratoire de Biologie-Vertébrés, Centre d'Orsay, Université Paris-Sud, 91405-Orsay, France and
[3]Département d'Anatomie et de Biologie Cellulaire, Unité de Biologie Intestinale, Faculté de Médecine, Université de Sherbrooke, Sherbrooke, Québec, Canada, J1H 5N4

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**Abstract** · 1. Intestinal brush border membranes have been isolated from *Rana catesbeiana* adult.

2. At the ultrastructural level a rather homogeneous preparation of membranous vesicles can be seen, and organelles other than the clear and dense vesicles could not be identified.

3. Negatively stained brush border fragments reveal the presence of particles on the outer surface of the membranes.

4. The membranous fraction is characterized by a high specific activity of trehalase, maltase, glucoamylase, γ-glutamyltranspeptidase and alkaline phosphatase.

## INTRODUCTION

Since 1960, the brush border of the intestinal epithelial cells of different mammal species was found to be a functionally unique subcellular organelle as well as a digestive-absorptive surface (Crane, 1975). Few studies have been done to characterize the intestinal brush border membranes of amphibian. In anuran small intestine, alkaline phosphatase (Chieffi & Carfagna, 1959; Botte & Buonanno, 1962; Brown & Millington, 1968; Kaltenbach *et al.*, 1978; Hourdry & Hugon, 1979) as well as maltase, trehalase, glucoamylase and γ-glutamyltranspeptidase have been detected (Hourdry *et al.*, 1979). However, sucrase and lactase activities are absent.

The small intestine of amphibian has also been used to study the mechanisms of absorption of sugars (Parsons & Prichard, 1965, 1971). Recently, there has been a surge of transport studies with isolated brush borders or brush border membranes (Hopfer *et al.*, 1976, 1978; Murer *et al.*, 1974, 1976; Tannembaum *et al.*, 1977; Kessler *et al.*, 1978). These isolated plasma membranes have proved to be a useful tool in evaluating cell membrane properties and transport systems. Similar studies using amphibian small intestine are nonexistent as no study has been devoted to isolate the intestinal brush border membranes from adult amphibian.

Recently, we have reported the successful isolation of intestinal brush border membranes from *Rana catesbeiana* tadpoles and confirmed the membrane-bound status of the different hydrolase activities found in the homogenates (Dauça *et al.*, 1979). In the present work, we have applied the brush border membrane isolation procedure to 2 yr old *Rana catesbeiana* and studied the enzymatic composition of the isolated brush border membranes as well as its ultrastructural features.

* To whom requests for reprints should be sent.

## MATERIALS AND METHODS

### *Animals*

Two year old *Rana catesbeiana* were purchased from Mogul, WI, U.S.A. They were kept at room temperature and fed minced meat.

### *Brush border membrane isolation*

The intestinal brush border fragments were isolated from the whole small intestine of adult *Rana catesbeiana* as described earlier for tadpole (Dauça *et al.*, 1979) using the Schmitz *et al.* (1973) isolation procedure. In brief, after decapitation, the intestine was removed, split open and washed in ice-cold saline. The scraped mucosa was homogenized and treated with calcium chloride ($CaCl_2$). Centrifugation of the calcium-treated homogenate ($H$) at 2000 *g* for 10 min yielded a pellet ($P_1$) and a supernatant ($S_1$). The supernatant was centrifuged at 20,000 *g* for 15 min to yield a pellet ($P_2$) containing brush border fragments and a final supernatant ($S_2$). $P_2$ was resuspended in ice-cold redistilled water. Proteins and enzymatic activities were determined in all subcellular fractions i.e. $H$, $P_1$, $S_1$, $P_2$ and $S_2$.

### *Assay methods*

Alkaline phosphatase and γ-glutamyltranspeptidase activities were measured according to Eichholz (1967) and Naftalin *et al.* (1969), respectively. Trehalase and maltase activities were assayed according to a modification by Lloyd & Whelan (1969) of Dahlqvist's (1964) method. Glucoamylase was measured using soluble starch as substrate according to Schlegel-Haueter *et al.* (1972). Proteins were assayed according to Lowry *et al.* (1951). The enzymatic activities were expressed as μmols of substrate hydrolysed per min (I.U.) per g protein. For each enzymatic activity, the recovery was calculated as well as the percentage of the total activity ($H$) in each subcellular fraction.

### *Electron microscopy*

Brush border fragments ($P_2$) were fixed according to Graham & Karnovsky (1966) as slightly modified by Dauça *et al.* (1979). Ultra-thin sections were examined in a

Philips 300 electron microscope. Brush border membranes were also examined by negative staining (Dauça *et al.*, 1979).

## RESULTS

### *Enzymatic composition of the brush border fragments*

All data are summarized in Table 1. The state of purity of the membranous fraction is estimated by the rise in specific activities of different enzymatic markers. In fraction $P_2$, 3% of the total proteins are found. Between 30–37% of the original γ-glutamyltranspeptidase, maltase, trehalase and glucoamylase activities are retained in fraction $P_2$. On the other hand 16% of the activity of alkaline phosphatase is noted in this fraction. Trehalase, maltase, glucoamylase and γ-glutamyltranspeptidase activities show an increase of 11–13 times in fraction $P_2$ in comparison with the specific activities present in the homogenate. Alkaline phosphatase specific activity is increased on the average 15 times in the membranous fraction. The recoveries for the different enzymatic activities range between 80–95% except for alkaline phosphatase (56%).

### *Electron microscopy*

The state of purity of the membranous fraction was also estimated by an ultrastructural study. Electron microscopy of fraction $P_2$ reveals a rather homogeneous preparation of membranous vesicles, although the vesicles are often still filled with electron dense material which presumably originated from the core of the microvilli (Fig. 1A). Very often intact

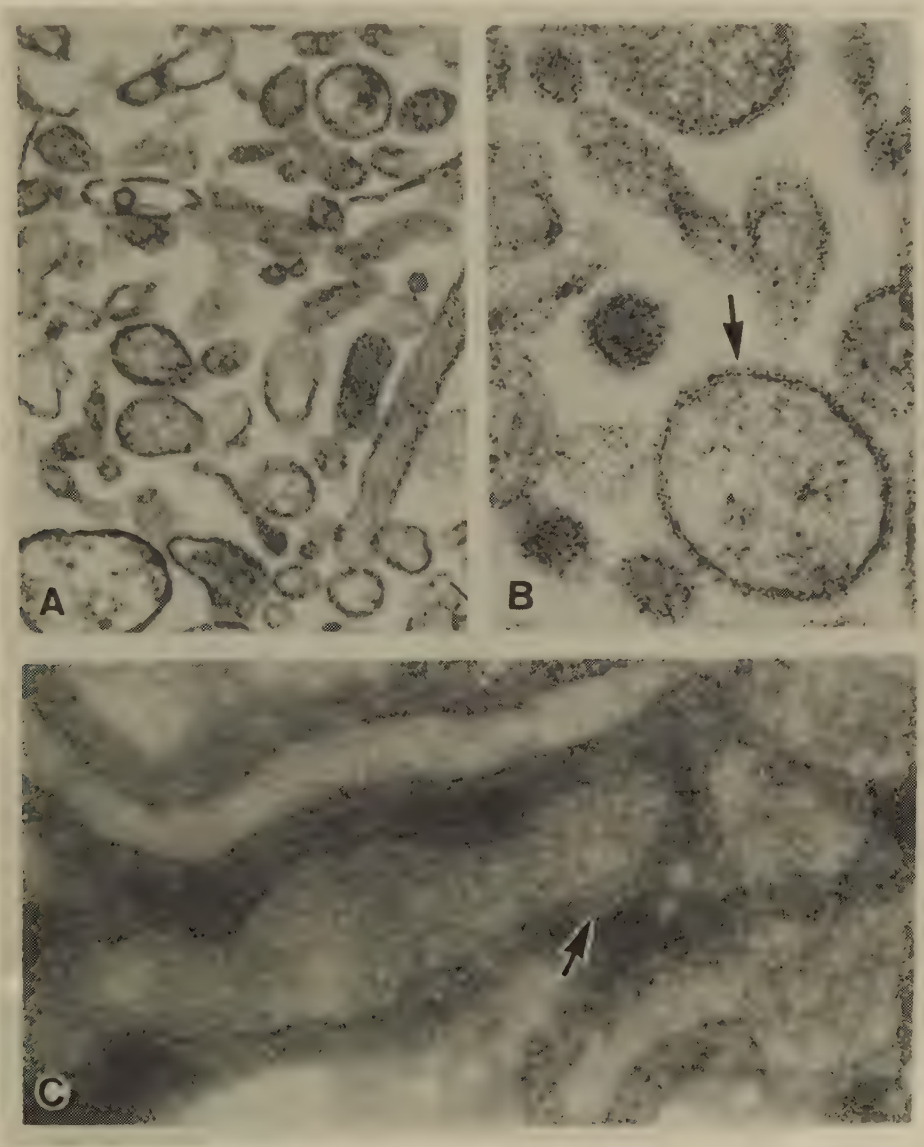


Fig. 1. Ultrastructural study of the microvillus membranes (Fraction $P_2$).

(A) This micrograph is the average representative of several micrographs which were obtained from 8 grids containing sections prepared from four different experiments. Fraction $P_2$ is generally composed of membranous fragments forming vesicles or tubules. Organelles other than the vesicles could not be identified. × 51,000.

(B) At higher magnification the trilaminar structure of the membrane is clearly seen as well as the granular appearance of the outer surface of the membrane (arrow). × 144,500.

(C) Particles or knob-like structures are observed covering the outer surface of the membrane (arrow) after negative staining. × 217,600.

Table 1. Enzymatic composition of microvillus membranes

| Fraction | Protein (%) | Alkaline phosphatase Sp. act.* | % | Glutamyltranspeptidase Sp. act. | % | Glucoamylase Sp. act. | % | Maltase Sp. act. | % | Trehalase Sp. act. | % |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *H* | 100 | 165 ± 18 (10) | 100 | 27 ± 1.8 (10) | 100 | 31 ± 2.3 (10) | 100 | 41 ± 1.8 (9) | 100 | 10 ± 0.9 (10) | 100 |
| $P_1$ | 44.7 ± 2.9 (10) | 121 ± 12 (10) | 32.6 ± 1.8 | 32 ± 1.6 (10) | 54.5 ± 3.6 | 22 ± 2.3 (10) | 30.5 ± 2 | 31 ± 2.8 (9) | 32 ± 2.1 | 9.2 ± 0.6 (10) | 43.1 ± 2.1 |
| $P_2$ | 3 ± 0.2 (10) | 2441 ± 430 (10) | 16.3 ± 1 | 328 ± 37 (10) | 36.1 ± 2.4 | 396 ± 48 (10) | 37.2 ± 2 | 477 ± 57 (9) | 34.6 ± 2.7 | 106 ± 17 (10) | 30.5 ± 1.5 |
| $S_2$ | 33.7 ± 1.9 (10) | 14 ± 1 (10) | 7.2 ± 1.2 | 4.3 ± 1 (10) | 4.9 ± 0.8 | 11 ± 1.4 (10) | 11.8 ± 0.9 | 21 ± 1.7 (9) | 17.4 ± 0.2 | 1.4 ± 0.3 (10) | 5.6 ± 1.5 |
| Recovery§ | 81.5 ± 4.6 | | 56.1 ± 3 | | 95.5 ± 5.5 | | 79.5 ± 4 | | 84 ± 5.6 | | 79.2 ± 3.2 |

* Sp. act.: Specific activity in I.U./g protein.
† Mean ± SE.
‡ Number of different adults studied.
§ Recoveries are means of the recoveries.

microvillus are seen. At high magnification the outer surface of the membrane has a granular appearance (Fig. 1B). Organelles other than the clear and dense vesicles could not be identified. Negatively stained brush border fragments reveal the presence of particles on the outer surface of the membranes (Fig. 1C), the inner surface of the membrane being free of any particles.

## DISCUSSION

The present study shows that it is possible to isolate a brush border membrane fraction from the small intestine of adult *Rana catesbeiana*. The brush border membranes observed at the ultrastructural level are similar to that obtained from Anuran tadpoles (Dauça *et al.*, 1979), chicken (Tilney & Moosecker, 1971), mice (Ballington & Nayudu, 1975), rat (Forstner *et al.*, 1968; Kessler *et al.*, 1978), hog (Maroux & Louvard, 1977) and man (Schmitz *et al.*, 1973). The granulated aspect of the outer surface of the membrane probably results from the numerous knob-like structures (particles) visualized by negative staining (Fig. 1C). These particles are probably in relation with the brush border hydrolase activities measured in the membranous fraction isolated from adult *Rana catesbeina*, such relationship being already established in mammals (Johnson, 1967; Benson *et al.*, 1971; Louvard *et al.*, 1975; Maestracci, 1976).

All the five enzymatic activities studied are indeed associated with the brush border membrane in adult *Rana catesbeiana* as demonstrated in anuran tadpole (Dauça *et al.*, 1979) and in mammals (Crane *et al.*, 1976). In fraction $P_2$ an 11–15 fold increase of the specific activities of trehalase, alkaline phosphatase, glucoamylase, maltase and $\gamma$-glutamyl transpeptidase over the specific activities present in the homogenates, is observed. These purification factors are higher than those found in tadpoles (9–13 fold) but similar to that reported for adult mammals (Schmitz *et al.*, 1973; Galand & Forstner, 1974; Kessler *et al.*, 1978; Raul *et al.*, 1978).

Enzyme recoveries are similar (i.e. 80–95%) for $\alpha$-glucosidases and $\gamma$-glutamyltranspeptidase, indicating that these enzymatic activities were not affected by the preparative technique. By contrast, only 56% of the alkaline phosphatase activity is recovered. A similar pattern has been described by Forstner *et al.* (1968) during the isolation of rat intestinal microvillus membranes. Therefore, alkaline phosphatase appears to be less suitable as brush border membrane marker in amphibian small intestine. Furthermore numerous studies have demonstrated that alkaline phosphatase activity can be visualized by histochemical means at the brush border membrane level but also on the basolateral membranes, Golgi, some lysosomes in the epithelial cells and even in the connective tissue (Chieffi & Carfagna, 1959; Brown & Millington, 1968; Kaltenbach *et al.*, 1978; Hourdry & Hugon, 1979).

In *Rana Catesbeiana* tadpole, 15–17% of the enzymatic activities measured in the homogenate are recovered in the membranous fraction (Dauça *et al.*, 1979). The present study shows that 30–37% of the enzymatic activities are recovered in $P_2$ fraction when isolated from the small intestine of *Rana catesbeiana* adult. This difference in the yield could be explained by a smaller loss of the activities in fraction $P_1$ and by a smaller percentage of enzymatic activities found in the final supernatant. The yields as well as the purification factors noted in adult *Rana catesbeiana* compare well with that reported in different adult mammals (Forstner *et al.*, 1968; Schmitz *et al.*, 1973; Stariata-Geribaldi *et al.*, 1977; Kessler *et al.*, 1978).

The specific activities of maltase, trehalase, glucoamylase and alkaline phosphatase noted in the intestinal mucosa of tadpole and adult *Rana Catesbeiana* do not change very much (Hourdry *et al.*, 1979; Dauça *et al.*, 1979). However, it appears that the specific activity of $\gamma$-glutamyltranspeptidase in adult *Rana catesbeiana* is 3–4 times higher than that observed in tadpole. Meister (1973) has proposed that $\gamma$-glutamyltranspeptidase acts as a membrane-localized transporter of aminoacids and possibly peptides and the recent studies of Garvey *et al.* (1976) on rat intestine support this concept. $\gamma$-glutamyltranspeptidase hydrolyses only $\gamma$-glutamic acid–peptide bonds found in a group of physiologically important peptides such as glutathione, folic acid and gluten (Cohen *et al.*, 1969). The present observations show that after metamorphosis when the animals are carnivorous and insectivorous (Hourdry & Dauça, 1977), the intestinal mucosa is well equipped for the hydrolysis of $\gamma$-carboxyl-amide bonds.

In conclusion, the intestinal brush border membranes can be isolated with success from adult *Rana catesbeiana* using the $Ca^{2+}$ precipitation procedure as developed by Schmitz *et al.* (1973) and as applied to tadpole small intestine (Dauça *et al.*, 1979). It is now possible to compare and to characterize the protein composition of amphibian intestinal brush border membrane during the metamorphosis.

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AMPHIBIAN INTESTINAL BRUSH BORDER MEMBRANES.
III. COMPARISON DURING METAMORPHOSIS OF THE PROTEIN, GLYCOPROTEIN AND ENZYME PATTERNS AFTER GEL ELECTROPHORETIC SEPARATION OF SDS-SOLUBILIZED MEMBRANES.

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# AMPHIBIAN INTESTINAL BRUSH BORDER MEMBRANES—III. COMPARISON DURING METAMORPHOSIS OF THE PROTEIN, GLYCOPROTEIN AND ENZYME PATTERNS AFTER GEL ELECTROPHORETIC SEPARATION OF SDS SOLUBILIZED MEMBRANES

M. DAUÇA[1,2], J. HOURDRY[2], J. S. HUGON[3] and D. MENARD[3]*

[1]Laboratoire de Biologie et Biochimie du Développement, Ecole Normale Supérieure, Grille d'Honneur-Parc de Saint-Cloud, 92211-Saint-Cloud, France
[2]Laboratoire de Biologie-Vertébrés, Centre d'Orsay, Université Paris-sud, 91405, Orsay, France
[3]Département d'Anatomie et de Biologie Cellulaire, Unité de Biologie Intestinale, Faculté de Médecine, Université de Sherbrooke, Sherbrooke, Québec, Canada J1H 5N4

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**Abstract**—1. Proteins associated with intestinal brush border membranes before and after metamorphosis of *Rana catesbeiana* were solubilized with sodium dodecyl sulfate (SDS) and separated by electrophoresis on acrylamide gels.

2. An increasing number of protein bands is observed in the brush border membrane protein patterns during the development of *Rana catesbeiana.*

3. The protein bands correspond to a heterogeneous group of polypeptides of molecular weights ranging from 28,000 to over 300,000 daltons.

4. Glycoproteins found in brush border membranes have high molecular weights. Protein bands that co-migrate with maltase, glucoamylase and alkaline phosphatase activities have been identified.

5. The relative mobility of alkaline phosphatase activity is always higher in tadpole's intestinal membrane than in adult's.

6. Trehalase and γ-glutamyltranspeptidase activities were inhibited by SDS and their positions on the gels remain undefined.

7. Brush border membrane hydrolytic activities are inhibited to a different extent depending on the developmental stage of *Rana catesbeiana.*

## INTRODUCTION

At climax of Anuran metamorphosis the intestinal primary epithelium degenerates and is replaced by a secondary epithelium folded into villi (Hourdry and Dauça, 1977). Even though the morphometric (Carver & Frieden, 1977) or histological modifications (Bonneville, 1963; Dournon & Chibon, 1974; McAvoy & Dixon, 1977; Dauça & Hourdry, 1977, 1978) occurring in Anuran amphibian intestine have been extensively studied, the enzymatic modifications taking place at the level of the brush border membrane have received little attention. Indeed, few studies have been done concerning the digestive hydrolases of the brush border in amphibia and their behavior during the metamorphosis (Parsons & Prichard, 1971; Lestage, 1976; Hourdry *et al.*, 1979). However, alkaline phosphatase activity has been relatively well studied either by histochemical (Chieffi & Carfagna, 1960; Brown & Millington, 1968; Kaltenbach *et al.*, 1977; Hourdry & Hugon, 1979) or biochemical means (Yanagasawa, 1954; Botte & Buonanno, 1962).

We have recently shown that it is possible to isolate intestinal brush border membranes from tadpole and adult *Rana catesbeiana* (Dauça *et al.*, 1979, 1980). These membranes contain maltase, glucoamylase, trehalase, alkaline phosphatase and γ-glutamyltranspeptidase activities but lack sucrase and lactase activities. As we have shown that these hydrolytic activities exhibit variations during the normal development of *Rana catesbeiana* (Hourdry *et al.*, 1979; Dauça *et al.*, 1979, 1980), the present investigation was undertaken to characterize and to compare the protein and glycoprotein compositions of intestinal brush border membranes during the metamorphosis.

## MATERIALS AND METHODS

### *Animals*

*Rana catesbeiana* were purchased from Mogul, WI, U.S.A. The study was done on three groups of animals: (1) tadpoles in premetamorphosis (stages I–VII according to Taylor and Kollros, 1946), (2) newly metamorphosed animals and, (3) adult *Rana catesbeiana*, 2 yr old.

### *Brush border membrane isolation*

After decapitation, the intestine was removed, slit lengthwise, washed with ice cold saline and the mucosa was scrapped off. When tadpoles or newly metamorphosed animals were studied, a pool of 2–4 animals was used for each experiment. The intestinal brush border membrane isolation procedure of Schmitz *et al.* (1973), as applied to amphibian intestine (Dauça *et al.*, 1979, 1980) was used. The

* To whom requests for reprints should be sent.

membranous fraction (fraction $P_2$) was resuspended in ice-cold redistilled water.

*Acrylamide gel electrophoresis*

Multiple-sample gel slabs were cast in a 4.5, 8% acrylamide monomer gradient in Tris sulfate buffer, pH 9.0, containing 0.1% sodium dodecylsulfate (SDS). After formation of wells with 8% gel, the solubilized membranes (1% SDS) were suspended in buffered sucrose, layered into the wells and capped with 8% gel. Electrophoresis (Ortec 4100 system) was performed using 0.065 M Tris–borate, pH 9.0, as upper and lower buffer, and a pulsed constant-power supply (280 V and 1.0 μF). The pulses per minute were raised from 75–300 over a period of 40 min and the electrophoresis was allowed to continue for a total run of 70 min (Preiser *et al.*, 1974). The upper buffer contained a trace of bromophenol blue and 0.1% SDS. Fifty micrograms of protein, as determined by the procedure of Lowry *et al.* (1951) were generally applied except for the study of glycoprotein when loads from 80–90 μg of membrane proteins were used. After completion of the electrophoresis, the slabs were removed and used for protein and glycoprotein staining as well as for enzymic determinations.

*Protein staining and destaining*

Parts of the slabs were fixed in 12.5% trichloroacetic acid, stained for 2 hr at 65°C in 0.07% Coomassie Brilliant Blue and destained in methanol:acetic acid:water (2:3:35).

*Glycoprotein staining*

The method of Glossmann & Neville (1971) was used.

*Histochemical study*

Gels were stained histochemically for alkaline phosphatase activity as described by Louvard *et al.* (1975).

*Assays*

The localization of hydrolytic activities after gel electrophoresis has been performed on 1 mm slices obtained with an electric gel slicer (Bio-Rad model 195) as described by Maestracci *et al.* (1975). The slices were homogenized in 0.5 ml of cold redistilled water or an appropriate buffer. After homogenization and centrifugation at 3000 rpm for 10 min the supernatants were assayed for various enzymatic activities. Trehalase and maltase activities, were determined according to a modification by Lloyd & Whelan (1969) of Dahlqvist's method (1964). Glucoamylase was assayed using soluble starch as substrate according to Schlegel-Haueter *et al.* (1972). Alkaline phosphatase activity and γ-glutamyltranspeptidase activity were assayed according to Eichholz (1967) and Naftalin *et al.* (1969), respectively.

Studies of the effects of SDS on the enzymatic activities of the intestinal brush border membranes were carried out on membrane fractions isolated from tadpoles and adults. Activities of the detergent-treated fractions were calculated as percentages of the activities found in the untreated.

*Determinations of apparent molecular weights*

Relative mobilities were calculated by comparison with the migration of the marker dye bromophenol blue. Proteins used as markers are listed in Table 1. The position of each marker relative to the bromophenol blue front was measured after staining and a plot of the logarithm of molecular weight versus relative mobility was drawn. The apparent molecular weights of the brush border proteins were then determined from this curve (Weber & Osborn, 1975).

The relative mobilities of the stained protein bands and those of the region of enzymic activities from the same slab gel were compared.

## RESULTS

*Log of molecular weight versus relative mobility*

Figure 1 shows the curve obtained when values of relative mobilities for different markers (Table 1) are plotted against their molecular weights. The electrophoretic system used stacks protein–SDS complexes over a mol. wt range of 24,000 to 335,000 daltons. In the range of 34,700–280,000 daltons a straight line can be drawn and six markers lie on the line with deviations in mol. wt less than 5% (regression coefficients: $a = -1.13$, $b = 5.52$; $r^2 = 0.98$). The points for hemocyanin (dimer), β-galactosidase and catalase show respective deviations of 12, 11 and 7% from this line. For molecular weights below 24,000 or above 335,000, the curve between their corresponding points was drawn by extrapolation. Thyroglobulin dimer does not enter the 8% gel.

*Purity of the membranous fraction*

Immediately after the intestinal brush border membrane isolation, the state of purity was determined using the alkaline phosphatase as a microvillous enzymatic marker. As shown in Table 2, the membranous fractions isolated from tadpoles, newly metamor-

Table 1. Proteins used as markers

| Protein | Code | Mol. wt × $10^{-3}$ | Source |
|---|---|---|---|
| Thyroglobulin (dimer) | 1a | 669 | Schwarz/Mann. |
| Thyroglobulin (monomer) | 1b | 335 | |
| Hemocyanin (tetramer) | 2a | 280 | |
| Hemocyanin (trimer) | 2b | 210 | Sigma |
| Hemocyanin (dimer) | 2c | 140 | |
| Hemocyanin (monomer) | 2d | 70 | |
| Uréase (trimer) | 3a | 240 | Sigma |
| Uréase (monomer) | 3b | 83 | |
| β-Galactosidase | 4 | 130 | Sigma |
| Phosphorulase a | 5 | 94 | Sigma |
| Catalase | 6 | 58 | Sigma |
| Pepsin | 7 | 34.7 | Sigma |
| Trypsinogen | 8 | 24 | Sigma |

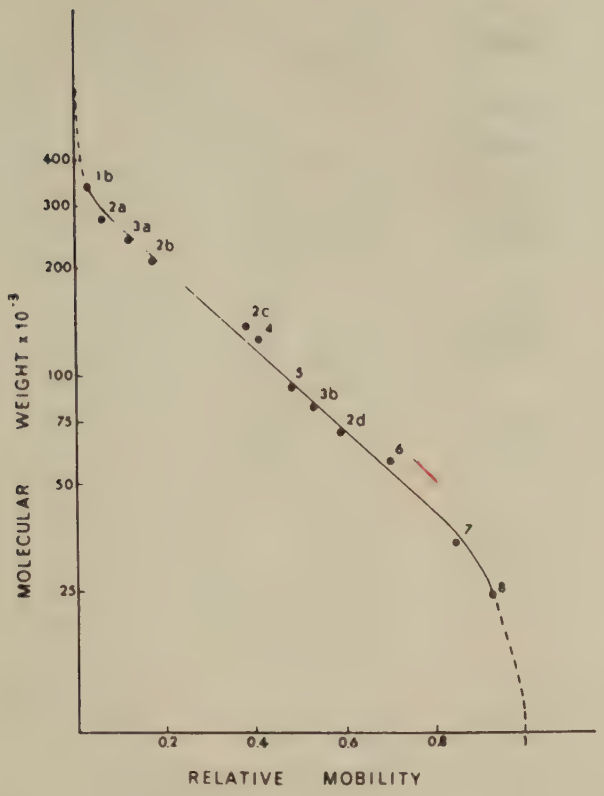


Fig. 1. Plot of the logarithm of the protein molecular weights of a series of protein–SDS complexes versus their relative mobilities. Numbers refer to protein code in Table 1. Electrophoresis was done as described under Materials and Methods. Each point represents the mean of nine determinations.

phosed and adult *Rana catesbeiana* contain 2–3% of the total proteins. The alkaline phosphatase activity shows an increase of 11–13 times in the $P_2$ fraction in comparison with the specific activity present in the homogenates. These data are in agreement with those reported earlier (Dauça *et al.*, 1979, 1980).

*Electrophoretic fractionation of brush border membrane proteins during development*

The protein patterns obtained for each studied stage appeared to be remarkably reproducible and revealed polypeptide heterogeneity of the intestinal brush border membrane of *Rana catesbeiana*. (Fig. 2). In tadpoles, 14 protein bands are noted with mol. wt ranging from 37,000–313,000 daltons. In newly metamorphosed animals 16 bands are found comparatively to 22 in adult *Rana catesbeiana* with mol. wt varying from 28,500–313,000 daltons.

The most prominent bands have high molecular weights. In tadpoles as well as in newly metamorphosed animals seven bands have a mol. wt higher than 150,000 daltons as compared to eight bands in adults. In this high molecular weight area, the metamorphosis does not provoke marked modification in the number of bands except for band 7 which is very faint in the newly metamorphosed animals as compared to tadpoles. The area including protein bands with mol. wt lower than 150,000 daltons contains more bands in newly metamorphosed animals. The adult protein pattern is slightly different from that of newly metamorphosed *Rana catesbeiana*. Indeed intensification of certain protein bands are noted while the appearance of others are observed particularly in the region of mol. wt lower than 150,000 daltons. In the high molecular weight area it seems that band 4 observed in newly metamorphosed animals corresponds to two bands in adult intestinal membrane: band 4a and 4b.

*Glycoprotein patterns*

In tadpole, newly metamorphosed and adult *Rana catesbeiana*, only glyco-proteins with molecular weight higher than 150,000 daltons are clearly visible.

Comparison of the membrane profile of gels stained for proteins and carbohydrates suggest that all the glycoproteins could be identified as membrane proteins. In tadpoles four major carbohydrates containing bands correspond to protein 1, 2, 4 and 5. A large Schiff positive band corresponds to protein 6 and 7, while a faint band is associated to protein 3. In newly metamorphosed and adult *Rana catesbeiana*, the first six protein bands are glycoproteins. However, a large carbohydrate containing band represents protein 4a and 4b in adult amphibian.

*Enzymic localization after gel electrophoresis*

*Histochemical study.* The use of a chromogenic substrate for the localization of alkaline phosphatase activity on acrylamide gel lead to the appearance, in a

Table 2. Purification of microvillus membranes (fraction $P_2$) in developing *Rana catesbeiana*

| Developmental stage | n‡ | Protein* % | Increase in† spec. act. |
|---|---|---|---|
| Tadpole (I.VII) | 16 | 2.5 ± 0.2 | 11.2 ± 0.7§ |
| Young (XXV) | 6 | 2.5 ± 0.6 | 11.5 ± 1.4 |
| Adult | 12 | 3.2 ± 0.2 | 13.3 ± 1.1 |

* Percentage of protein in fraction $P_2$ compared to the original amount of the homogenate.

† Increase in alkaline phosphatase specific activity in fraction $P_2$ over the homogenate.

‡ Number of experiments.

§ Mean ± SE.

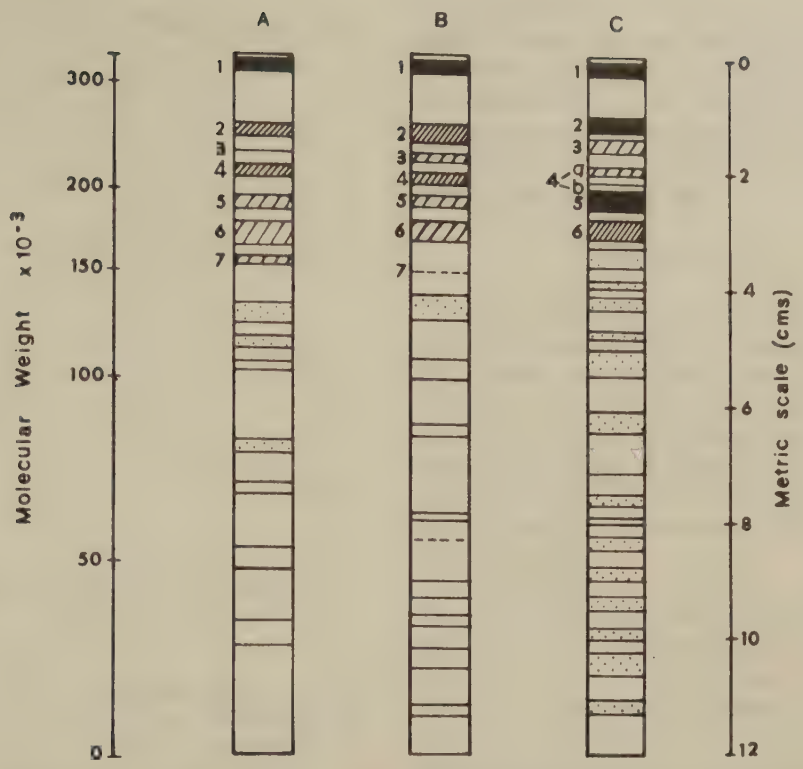


Fig. 2. Diagram comparing the acrylamide gel electrophoresis band patterns obtained from the intestinal brush border membranes of tadpole (A), young (B) and adult (C) *Rana catesbeiana*. The diagram summarizes the observations made on 8 independent electrophoresis for A and C and of 4 electrophoresis for B.

few minutes, of a large red band. As shown in Fig. 3, the relative mobility ($R_f$) of this enzymatic activity is higher in tadpole intestinal membrane than in adult's. The mean $R_f$ values are respectively 0.26–0.30 for tadpole, 0.24–0.28 for newly metamorphosed animals and 0.23–0.27 for adults. In tadpoles, this enzymatic activity is associated mainly to band 7 and partially to band 6 while in newly metamorphosed and adult *Rana catesbeiana* it is associated to band 6.

*Biochemical study*

*Alkaline phosphatase.* Six independent experiments with tadpoles show an $R_f$ value of 0.27–0.30 for alkaline phosphatase activity with an apparent molecular weight of 152,000 ± 3,000. This activity is associated to protein band 7. In newly metamorphosed amphibian (4 experiments) an $R_f$ value of 0.24–0.27 is observed as compared to 0.25–0.28 in adult *Rana catesbeiana* (6 experiments). In both cases, the alkaline phosphatase activity is associated to protein band 6. In adult, the apparent mol. wt is 167,000 ± 3,000.

*Maltase–glucoamylase.* Maltase activity is mainly associated to protein band 2 in tadpole (mol. wt 245,000), in newly metamorphosed (mol. wt 245,000) and in adult *Rana catesbeiana* (mol. wt 255,000). A low activity is detected in protein band 5 in tadpole (mol. wt 190,000), in protein band 4 (mol. wt 210,000) and protein bands 4a, 4b (mol. wt 210–220,000) in newly metamorphosed and adults, respectively.

At all developmental stages, glucoamylase activity is mainly associated to protein band 2 even though a low but detectable activity is also observed with the faster running maltase activity.

*Trehalase and γ-glutamyltranspeptidase.* It has been impossible to detect any trehalase or γ-glutamyltranspeptidase activity after gel electrophoresis. Protein band 1 probably corresponds to the beginning of the

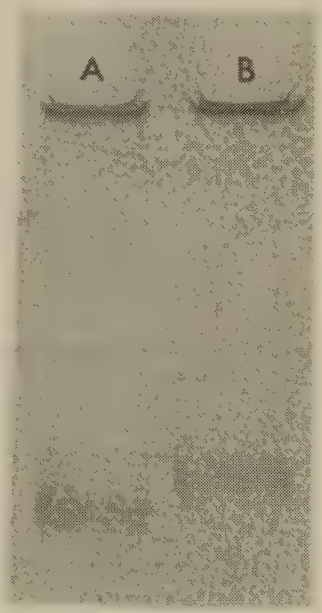


Fig. 3. Localization of alkaline phosphatase (Al. Pase) activity after gel electrophoretic separation of intestinal brush border membranes for tadpole (A) and adult (B) *Rana catesbeiana*. The relative mobility of tadpole Al. Pase activity is higher than the adult's.

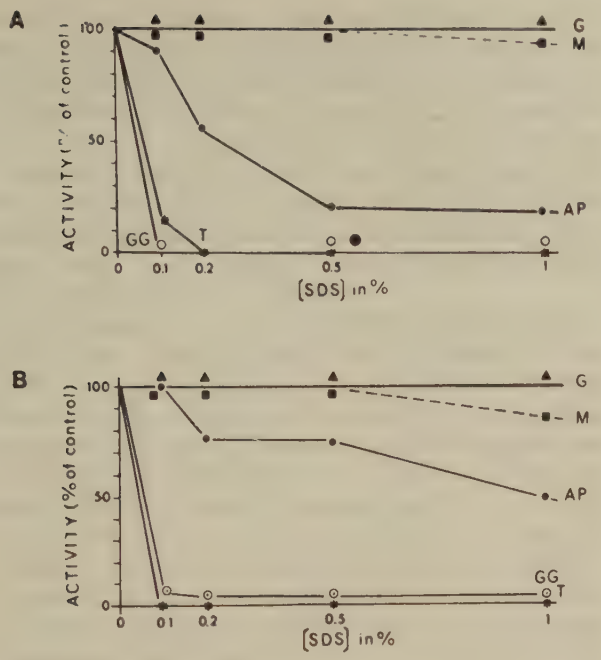


Fig. 4. The effects of sodium dodecylsulfate (SDS) on brush border enzyme activities. Aliquots of tadpole (A) adult (B) *Rana catesbeiana* brush border fragments were treated with SDS at the concentrations shown in the figure. Enzymic assays were performed 4 hr after the addition of the detergent. The enzyme activities in the detergent-treated fraction were compared with that of the same amount of the untreated preparation. Each point in the figure represents the mean of three determinations. AP, alkaline phosphatase; M, maltase; G, glucoamylase; T, trehalase; GG, γ-glutamyltranspeptidase.

8% gel and alkaline phosphatase, maltase and glucoamylase activities have been detected.

*Enzyme inactivation as a function of SDS concentration*

The inability to detect any trehalase and γ-glutamyltranspeptidase activity after gel electrophoresis lead us to study the effects of different concentrations of SDS on enzymatic activities. Figure 4 illustrates the effects of increasing concentrations of SDS on brush border enzymatic activities in tadpoles (Fig. 4A) and in adults (Fig. 4B) during 4 hr. This period of time has been retained as it roughly corresponds to the period of time between the layering of the protein samples into wells and the end of the enzymatic assays. Trehalase and γ-glutamyltranspeptidase activities are already completely inhibited by 0.2% SDS. A very low activity (less than 10%) is still detectable with 0.1% SDS, which concentration however does not ensure a complete solubilization of membranes. Alkaline phosphatase activity is decreased, and it appears that tadpole's alkaline phosphatase is more sensitive to SDS. However, even though there is a decrease of activity, the remaining activity is sufficient to allow its localization on gel. Finally, maltase and glucoamylase activities are not inhibited to a significant extent by SDS.

*Enzyme inactivation as a function of time of incubation with SDS*

Intestinal membranes isolated from tadpole and adult *Rana catesbeiana* were incubated with SDS (1% final) for 20 min to 6 hr (Figs 5A and 5B). It appears that trehalase and γ-glutamyltranspeptidase activities are rapidly inactivated, explaining why those activities are undetectable after gel electrophoresis. Alkaline phosphatase activity is partially inactivated especially in tadpoles. Again maltase and glucoamylase are almost unaffected by SDS.

## DISCUSSION

The use of SDS-polyacrylamide gel electrophoresis has been successful in the analysis of the protein, glycoprotein and enzymic composition of human (Maestracci *et al.*, 1973, 1975), hamster (Critchley *et al.*, 1975), guinea-pig (Green & Hadorn, 1977), rat (Alpers, 1972; Kim & Perdomo, 1974; Seetharam *et al.*, 1977), mouse (Billington & Nayudu, 1973) and chick (Tilney & Mooseker, 1971) intestinal brush border membranes.

Solubilization with SDS and subsequent electrophoresis fractionation show the heterogeneity of proteins in the intestinal brush border membrane isolated from tadpole, young and adult *Rana catesbeiana*. Moreover, a progressive increase in the number of protein bands is noted during the development. Although no technique can be used alone for the absolute determination of the number of polypeptides because of possible overlaps (Kobyla *et al.*, 1972), 14, 16 and 22 bands, at least, were present in tadpole, newly metamorphosed and adult *Rana catesbeiana*, respectively. The number of protein bands observed for adult bullfrog is relatively similar to that reported for dog (Starita-Geribaldi *et al.*, 1977) and man (Maestracci *et al.*, 1973). On the other hand, 10–15 protein or subunits were found in chicken (Tilney & Mooseker, 1971), rat (Alpers, 1972), hamster (Critchley *et al.*, 1975) and mouse (Billington & Nayudu,

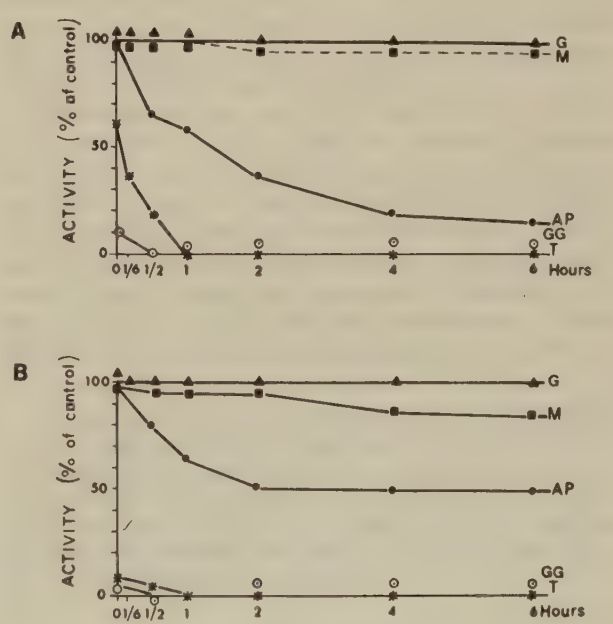


Fig. 5. The effects of 1% sodium dodecylsulfate (SDS) on brush border enzyme activities. Brush border fragments of tadpole (A) and adult (B) *Rana catesbeiana* were incubated with and without 1% SDS. Aliquots were removed for analysis of enzyme activities immediately and at different time intervals as indicated in the figure. Enzymic determinations and abbreviations are the same as for Fig. 4.

1973). Those differences could be attributed either to species variations or to electrophoretic conditions. In these studies, the gels were polymerized in presence of SDS and the electrophoresis carried out in a continuous system. In our experiments as well as for dog (Starita-Geribaldi *et al.*, 1977), man (Maestracci *et al.*, 1973) and rat (Hauri & Green, 1978), SDS was not present in the gel, and because a discontinuous system was used, more highly resolved patterns were obtained (Kobyla *et al.*, 1972).

The molecular weights of the polypeptide chains range from 28,000 to over 300,000 daltons. Those molecular weights found for amphibian intestinal brush border proteins are in agreement with those reported for mammal intestinal brush border proteins including man (Maestracci *et al.*, 1973). During the development of *Rana catesbeiana*, there is an increase of the low molecular weight protein bands (Fig. 2). The procedure used in this study, as well as for others, for the isolation of the brush border membrane fragments give a fraction composed mainly of membranes and some fibrillar components of the microvillar core (Dauça *et al.*, 1979, 1980). Further purification steps would be needed to remove the fibrillar core proteins (Maestracci *et al.*, 1973). Most proteins of the core have a molecular weight lower than 50,000 daltons. So the noted increase of the low molecular weight protein bands during the development of *Rana catesbeiana* could be related to the replacement of the primary epithelium by the secondary epithelium folded into villi where enterocytes would exhibit a more developed brush border. No experiment was done in this direction as we were mainly interested in the high molecular weight protein bands in relationship to enzymatic activities.

The present study reports the identification of high molecular weight protein bands that co-migrate with maltase, glucoamylase and alkaline phosphatase activities after electrophoresis on SDS/polyacrylamide gels under non-reducing conditions. Several studies have demonstrated that the activities of many intestinal brush border enzymes can survive polyacrylamide–gel electrophoresis in the presence of SDS, thereby enabling the appropriate bands to be identified afterwards (Maestracci *et al.*, 1975; Critchley *et al.*, 1975; Green & Hadorn, 1977; Flanagan & Forstner, 1979). In amphibian, maltase and glucoamylase activities are localized in the same protein bands having a mol wt of 245,000 and about 200,000 daltons. Maltase/glucoamylase is the largest component of the microvillus membrane in mammal's intestine (Maestracci *et al.*, 1975; Critchley *et al.*, 1975; Flanagan & Forstner, 1979) and it appears that the same observation can be made in amphibian intestine (Fig. 2). However, the molecular weight noted for amphibian maltase/glucoamylase complex is somewhat lower than that reported for human complex (mol. wt 440,000) where the activity was associated with one protein band (Maestracci *et al.*, 1975). Recently, Flanagan & Forstner (1979) reported that rat maltase/glucoamylase has a molecular weight of 500,000 daltons and during electrophoresis the complex could be partly dissociated giving rise to several protein bands enzymatically active.

During the development of *Rana catesbeiana*, alkaline phosphatase exhibits an interesting pattern. In adult, Al. Pase activity co-migrate with a protein band having a mol. wt of 167,000. A similar molecular weight is noted in mammal's intestine (Maestracci *et al.*, 1975). However, the relative mobility of Al. Pase activity is always faster in tadpoles as compared to those observed in young and adult bullfrogs. Using cytochemical means, Kaltenbach *et al.* (1977) reported clear differences in the affinity of alkaline phosphatase for different substrates in small intestine of tadpole and adult *Rana pipiens*. It is quite possible that intestinal alkaline phosphatase of *Rana catesbeiana* exhibits different isoenzymes during the post-embryonic life as demonstrated for Xenopus larvae (Maekawa & Yamana, 1975). Therefore, it appears that maturation process occurs for alkaline phosphatase during the development of Amphibia. Such modifications have been reported in mammals (Moog & Yek, 1973; Raul *et al.*, 1977; Simon *et al.*, 1979; Benham & Harris, 1979).

After gel electrophoresis is has been impossible to localize trehalase and $\gamma$-glutamyltranspeptidase activities. Indeed, from the study on the effects of SDS on brush border enzyme activities, it is clear that these two enzymic activities are very sensitive to SDS in tadpole as well as in adult *Rana catesbeiana* (Figs 4, 5). The complete inhibition of T and $\gamma$-GT activities by SDS observed in amphibian is in contrast to the observed behaviour for these enzymes in mammal's intestine. Indeed, even though these enzymic activities are greatly inhibited by SDS, residual activities (10–20%) can be detected enabling the appropriate protein bands to be identified (Critchley *et al.*, 1970; Maestracci *et al.*, 1975). Maltase/glucoamylase complex is slightly inhibited by the detergent, either in tadpole or in adult *Rana catesbeiana*. The same observation has been reported in human small intestine (Maestracci *et al.*, 1975). Mammal intestinal Al. Pase is almost unaffected by SDS. On the other hand, amuran intestinal Al. Pase activity is inhibited to some extent by SDS, the degree of inhibition depending on the developmental stage (Figs 4, 5).

Differences in the sensitivity towards SDS of tadpole and adult *Rana catesbeiana* intestinal alkaline phosphatase, as well as for the other brush border hydrolytic activities between amphibian and mammals have been observed. These differences could suggest either a structural modification and/or an alteration in the microvillous membrane integration not only during the post-embryonic development of amphibians but also during the evolution of vertebrates.

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DEVELOPMENT OF PEROXISOMES IN AMPHIBIA.

I. CYTOCHEMICAL AND BIOCHEMICAL STUDIES ON THE SMALL INTESTINE.

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# DEVELOPMENT OF PEROXISOMES IN AMPHIBIAN

## I. CYTOCHEMICAL AND BIOCHEMICAL STUDIES ON THE SMALL INTESTINE

M. DAUÇA[1,3], R. CALVERT[2], D. MENARD[2],
J.S. HUGON[2], and J. HOURDRY[3]

1. Laboratoire de Biologie et Biochimie du Développement
Ecole Normale Supérieure
Grille d'Honneur - Parc de Saint-Cloud
92210 - Saint-Cloud - France.

2. Département d'Anatomie et de Biologie Cellulaire
Unité de Biologie Intestinale
Faculté de Médecine, Université de Sherbrooke
Sherbrooke, Québec, Canada J1H 5N4.

3. Laboratoire de Biologie-Vertébrés
E.R.A. 902
Centre d'Orsay - Université Paris-Sud
91405 - Orsay - France.

## ABSTRACT

The ontogeny of intestinal microperoxisomes was studied by cytochemical and biochemical methods during the development of two anuran amphibian species (Rana catesbeiana and Alytes obstetricans). The catalase activity was localized after incubation in DAB-medium and studied biochemically by a spectrophotometric method. Microperoxisomes were found to be present in the intestinal epithelial cells of these amphibians. The primary enterocytes of aquatic larvae showed very few microperoxisomes. After metamorphosis the absorptive cells of the new secondary epithelium exhibited a drastically increased number of microperoxisomes per cell section. These organelles showed circular profiles and had a diameter ranging from 0.36 to 0.40 µm. The intestinal catalase activity was low in the tadpoles, increased steadily after metamorphosis and peaked in the adults. Moreover at each developmental stage, the proximal segments always exhibited the highest catalase activity. The present study shows that the number of microperoxisomes as well as the catalase activity increase with the maturation of the intestinal epithelial cells in developing anuran amphibians.

# INTRODUCTION

Peroxisomes, first described in the cytoplasm of mouse proximal tubules (Rhodin, '58) and in rat hepatocytes (Rouiller and Bernhard, '56), were recognized following numerous investigations as a defined cell organelle (de Duve and Baudhuin, '66). Recently, Böck et al. ('80) published an excellent monograph on peroxisomes and related particles in which an exhaustive review of their distribution in mammalian and non-mammalian tissues was presented. Few studies have been done concerning the presence and/or the development of peroxisomes in different amphibian tissues. Therefore, an investigation was undertaken to study the ontogeny of peroxisomes in the small intestine, liver, pancreas and kidney during the development of two species of amphibian namely Rana catesbeiana and Alytes obstetricans.

During metamorphosis, the small intestine of anuran amphibians is drastically modified: its overall length decreases dramatically and the primary epithelium is completely replaced by a new epithelial layer exhibiting villi (Bonneville and Weinstock, '70; McAvoy and Dixon, '77; Hourdry and Dauça, '77; Dauça and Hourdry, '78). Furthermore these alterations are accompanied by a transformation of the herbivorous tadpole into a carnivorous juvenile. In previous work, we have analyzed the modifications of the intestinal brush border membrane hydrolytic activities during this period (Hourdry et al., '79; Hourdry and Hugon, '79; Dauça et al., '79, '80a, b, '81). In this first of a series of two papers, we report the presence and the developmental pattern of microperoxisomes in the intestinal epithelial cells. Herein, the term microperoxisome will be used instead of peroxisome on a purely

morphological basis: in the intestine, these organelles lack nucleoids and they have a smaller diameter than that of peroxisomes in liver and kidney.

## MATERIALS AND METHODS

Animals. Two anuran amphibian species were used: Rana catesbeiana tadpoles and adults were purchased from McNally (Wisconsin, USA), and Alytes obstetricans tadpoles and adults were obtained from small ponds in South of France. Young juveniles were produced by keeping early spring larvae at 20-25°C. Stages were determined according to Taylor and Kollros ('46).

Cytochemistry. The small intestine was rapidly excised, and pieces of the mucosa were selected between the entrance of the bile duct and the rectum. Samples were fixed during 30 min in a cold solution of 2.8% glutaraldehyde buffered with 0.1M sodium cacodylate and rinsed in 0.2M sodium cacodylate buffer; 40-µm-thick frozen sections were made immediately and incubated at pH 9.7, according to the method described by Novikoff et al. ('72), during 3 hrs. The incubation medium was renewed after 90 min of incubation. Incubated sections were postfixed in osmium tetroxide during 45 min, dehydrated and embedded in Epon. Thick Epon sections were stained during 1 min at 70°C with toluidine blue (1%) and sodium borate (1%). Thin sections were stained with uranyl acetate only or double stained with uranyl acetate and lead citrate (Reynolds, '63), and were examined in a Philips EM 300 or in a Jeol JEM 100B electron microscope.

Morphometry. The diameters of the microperoxisomes were measured on electron micrographs of 10 cells per developmental stage. At each stage, the number of microperoxisomes per cell section was evaluated in 20 cells from 2 animals by direct observation with the electron microscope. The standard deviation was not calculated because the surface of the cells

was not considered.

Biochemical methods. After decapitation, the intestine was removed and immersed in ice-cold M/15 phosphate buffer, pH 7.0. At each developmental stage, the gut was divided in two equal parts. The intestinal segments were slit lengthwise, washed in buffer and blotted dry. The mucosa was scrapped off with microscope slides, weighed and homogenized in 10 vol of ice-cold phosphate buffer with a glass-Teflon homogenizer. After homogenization, aliquots were taken for protein assays according to Lowry et al. ('51). Then, Triton X-100 was added to the homogenates to a final concentration of 1% as previously reported (Calvert and Ménard, '78). The homogenate was centrifuged at 5000 rpm/min during 10 min and the catalase activity was measured in the supernatant by the spectrophotometric method of Lück ('65). The enzymatic activity was expressed as units of catalase per g protein.

## RESULTS

Cytochemical observations. The intestine of premetamorphosis larvae of Rana catesbeiana and Alytes obstetricans (stages X - XII) is long and spiral; the primary epithelium is always devoid of villi. The stem cells of the secondary epithelium are already located at the base of the primary epithelium but they are difficult to identify. At this stage, DAB-positive microperoxisomes are not observed frequently and they have a mean diameter of 0.36 ± 0.01 µm in Rana catesbeiana larvae,and of 0.26 ± 0.01 µm in Alytes obstetricans larvae.

In the young juveniles adapted to their new terrestrial habitat, DAB-positive structures are easily identified in thick sections (Fig. 1). Most microperoxisomes are seen in the supra-nuclear region of secondary epithelial cells (Fig. 2). The strongly DAB-positive structures have circular profiles and they often form clusters (Fig. 3). Continuities between their unit membrane and that of the smooth endoplasmic reticulum are not obvious. The number of microperoxisomes per section of secondary epithelial cells located in the proximal segments of the small intestine increases in both species (Fig. 4). However the increase is far more important in Alytes obstetricans juveniles but the animals were 2-month-old when Rana catesbeiana juveniles were 2-week-old. During this period, the mean diameter of microperoxisomes measures 0.30 ± 0.01 µm in Rana catesbeiana and 0.38 ± 0.01 µm in Alytes obstetricans.In adults, the number of microperoxisomes per cell section increases only in Rana catesbeiana (Fig. 4). With the light microscope, microperoxisomes are identified in the supranuclear region of the secondary epithelial cells (Fig. 5); very few are located basally. In the supranuclear region it

seems that the highest concentration of DAB-positive structures is found closer to the nucleus or Golgi region than near the terminal web (Fig. 6). Most microperoxisomes remain circular in adults but in a few instances tubular microperoxisomes are present and occasionally their membrane is seen in continuity with that of the smooth endoplasmic reticulum (Fig. 7). At this stage, microperoxisomes have a mean diameter measuring 0.41 ± 0.02 μm in Rana catesbeiana and 0.36 ± 0.02 μm in Alytes obstetricans.

Biochemical observations. Catalase activity has been determined only in Rana catesbeiana since enough tissue can be obtained from a single animal at any studied developmental stage. In the first study, catalase activity has been analyzed in the proximal and distal halves of the small intestine. In tadpoles as well as in the adult bullfrogs, the proximal parts always exhibit the highest catalase activity (Table 1). The developmental changes of catalase activity in the proximal part of the small intestine are summarized in table 2. A significant decrease of activity is noted at the peak of the metamorphosis (tadpole stages XXIV-XXV) when the primary epithelium is being replaced by the secondary epithelium. In newly metamorphosed animals (2-week-old) a significant increase of catalase activity is noted when compared to previous stage. However, the activity is still significantly lower than that observed in the adult bullfrogs.

## DISCUSSION

This investigation reports for the first time the presence of DAB-positive microperoxisomes with a mean diameter of 0.36 to 0.40 µm in intestinal epithelial cells of two species of anuran amphibians. Microperoxisomes were identified by incubation of tissue sections in alkaline media containing DAB and hydrogen peroxide, thus demonstrating the peroxidatic activities of catalase (Novikoff et al., '72). This method and that of Novikoff and Goldfischer ('69) have been applied successfully without modification to amphibian tissues such as pancreas (Trandaburu, '80), interrenal cells (Berchtold, '75) and thyroid (Picheral, '72).

Peroxisomes are widely distributed in mammalian as well as in non-mammalian tissues (see review, Böck et al., 80). At the intestinal level, Novikoff and Novikoff ('72) reported large numbers of microperoxisomes in the small intestinal epithelial cells of adult rat and guinea pig. Particulate fractions rich in catalase have been separated from homogenates of small intestinal epithelium of mammals and fishs(Connock and Pover, '70; Peters, '70; '76; Connock, '73; Temple et al., '79). In amphibians, the proximal part of the gut always exhibits the highest catalase activity at all developmental stages. This distribution along the small intestine is similar to that reported in mouse (Calvert and Ménard, '78).

The ontogeny of peroxisomes and microperoxisomes in differentiating tissues has been mainly studied in liver and kidney of rat, mouse and chicken (Holmes and Masters, '78; Böck et al. '80). Few studies have been done on the ontogeny of microperoxisomes in the small intestinal

tissue (Pipan and Psenicnik, '75; Calvert and Ménard, '78). The small intestine of amphibians represents an interesting model since during metamorphosis the intestine is drastically modified, its mucosa being completely replaced by a new epithelial layer (Bonneville, '63; McAvoy and Dixon, '77; Hourdry and Dauça, '77; Dauça and Hourdry, '78). The present study shows that the new intestinal epithelial cells after metamorphosis exhibit more microperoxisomes as well as an increased catalase activity as compared to the primary epithelium of the tadpole. This observation is in agreement with that reported in mouse indicating that the number of microperoxisomes increases with the maturation of the absorptive cells (Calvert and Ménard, '78). Indeed, in anuran amphibian small intestine, the new epithelial cells observed after metamorphosis derived from stem cells (Dauça and Hourdry, '78). After intense proliferation, the new epithelial cells will differentiate: a new brush border will appear (Bonneville and Weinstock, '70) with all the digestive hydrolases related to this organelle (Hourdry et al. '79; Dauça et al. '79, '80a, '81) along with an increase of the number of microperoxisomes. In Rana catesbeiana, catalase activity and the number of microperoxisomes will still increase from juvenile (2-week-old) to adult stage (2-year-old). We do not know if this increase takes place in a short period of time or occurs throughout the entire developmental process. However, since more microperoxisomes are noted in Rana catesbeiana and Alytes obstetricans after metamorphosis and since no further increase is observed in intestinal epithelial cells of 2-month-old Alytes obstetricans as compared to adults (figure 4), we postulate that the modifications in number of microperoxisomes and in catalase activity occur during the first two months following metamorphosis.

The biological signification of the increase of microperoxisomes and of catalase activity during the development of the small intestine is not clear. In anuran amphibians, the metamorphosis leads to a change of habitat as well as a drastic modification of alimentation. Indeed, these animals become carnivorous and the microperoxisomes could be involved in lipid metabolism as suggested in mammals (Reddy, '74; Reddy et al. '77). It is interesting to point out that the plasma lipoprotein concentrations increase during post-embryonic development of amphibians (Nagano et al. '75).

In conclusion, the present study reports the presence of microperoxisomes in intestinal epithelial cells of anuran amphibian and shows that the number of microperoxisomes as well as the catalase activity increase with the maturation of the absorptive cells. The developmental patterns of this organelle is being studied in liver, kidney and pancreas in order to determine if the pattern observed in the small intestine could be generalized to all tissues.

## ACKNOWLEDGEMENTS

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TABLE 1

CATALASE ACTIVITY IN THE PROXIMAL AND DISTAL HALVES OF THE INTESTINE OF RANA CATESBEIANA

| Stage of development | Catalase activity[a] | | p[c] |
|---|---|---|---|
| | Proximal half | Distal half | |
| Tadpole (stages X-XII) | 2326.4 ± 278.1 (5)[b] | 915.9 ± 197.4 (6) | 0.005 |
| Adult (2 year-old) | 5892.7 ± 876.5 (8) | 2758.2 ± 325.0 (8) | 0.005 |

a. Mean ± SEM of catalase activity expressed as units of catalase/g of protein.

b. Number of animals.

c. By student's t test.

TABLE 2

CATALASE ACTIVITY IN THE PROXIMAL HALF OF THE INTESTINE OF DEVELOPING RANA CATESBEIANA

| Stage of Development | N[a] | Catalase activity[b] | p[c] |
|---|---|---|---|
| Tadpole (stages X-XII) | 12 | 2370.1 ± 148.5[1] | 1,2 < 0.025<br>1,3 < 0.025 |
| Tadpole (stages XXIV-XXV) | 9 | 1682.5 ± 246.6[2] | 2,3 < 0.005 |
| Juvenile (2 week-old) | 8 | 3310.2 ± 379.5[3] | 3,4 < 0.01 |
| Adult (2 month-old) | 8 | 5892.7 ± 876.5[4] | |

a Number of animals

b Mean ± SEM of catalase activity expressed as units of catalase/g of protein.

c By student's t test.

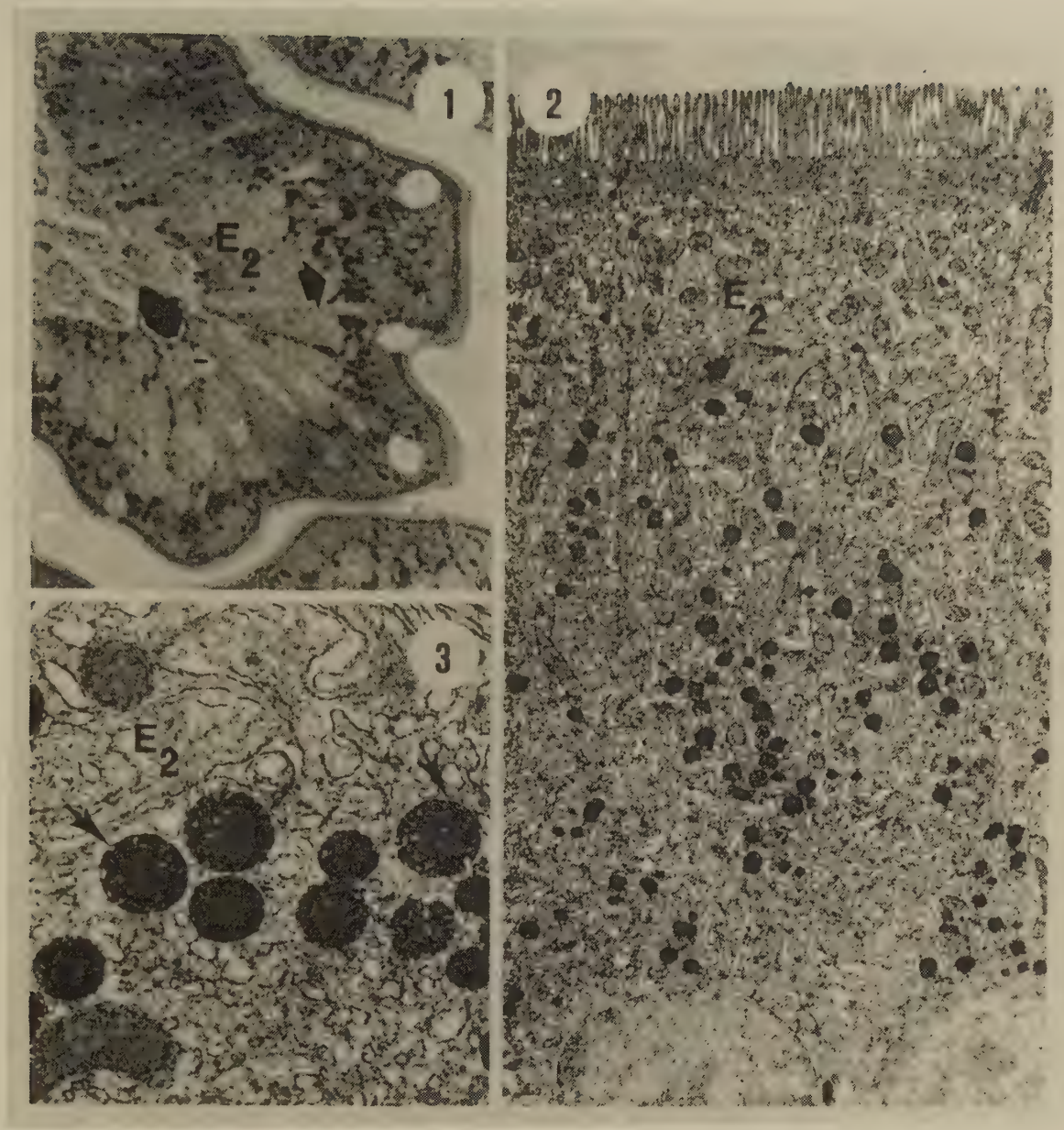


Fig. 1 Light micrograph of intestinal villi of a Rana catesbeiana juvenile. DAB-positive microperoxisomes (arrow) are numerous in the supranuclear region of the secondary enterocytes ($E_2$). Very few are located in the basal area. X 200.

Fig. 2 In Alytes obstetricans juveniles, microperoxisomes are concentrated in the lower two thirds of the supranuclear area in the secondary enterocytes ($E_2$). X 5,400.

Fig. 3 At higher magnification, most microperoxisomes show a circular profile. Their membrane may be identified easily (arrows) and continuities with the smooth endoplasmic reticulum are not frequent. X 25,300.

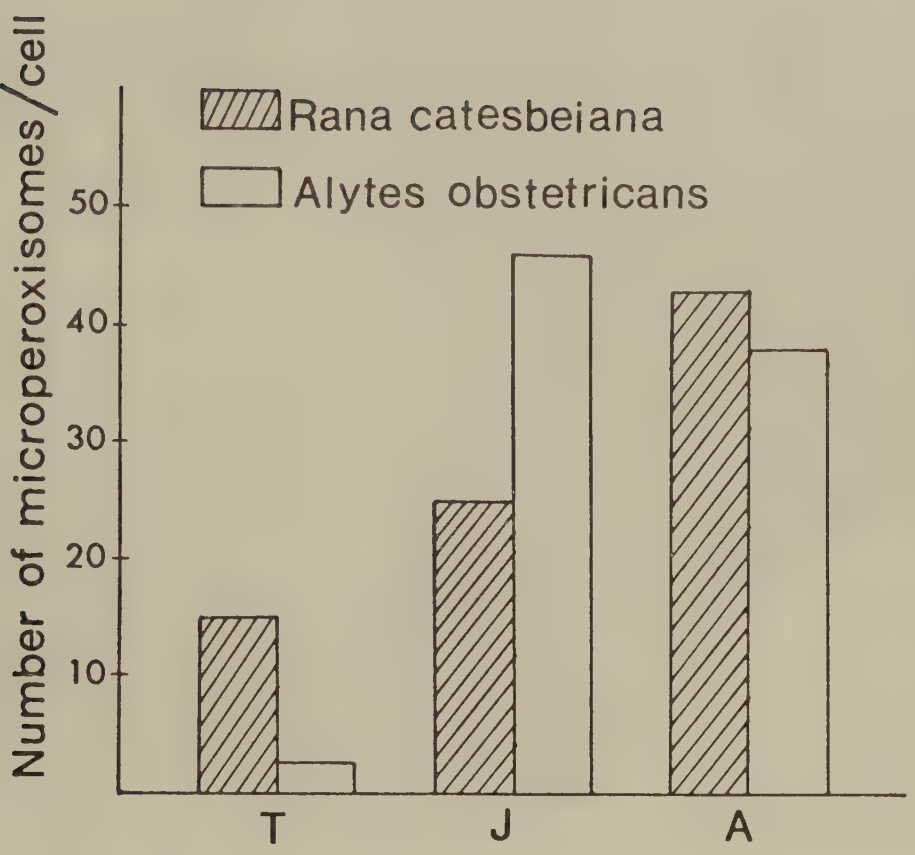


Fig. 4 This figure illustrates the mean number of microperoxisomes per cell section of enterocytes located in the proximal segments of the small intestine during amphibian development. T, tadpoles (stages X-XII); J, juveniles (2-week-old for Rana catesbeiana and 2-month-old for Alytes obstetricans); A, 2-year-old adults

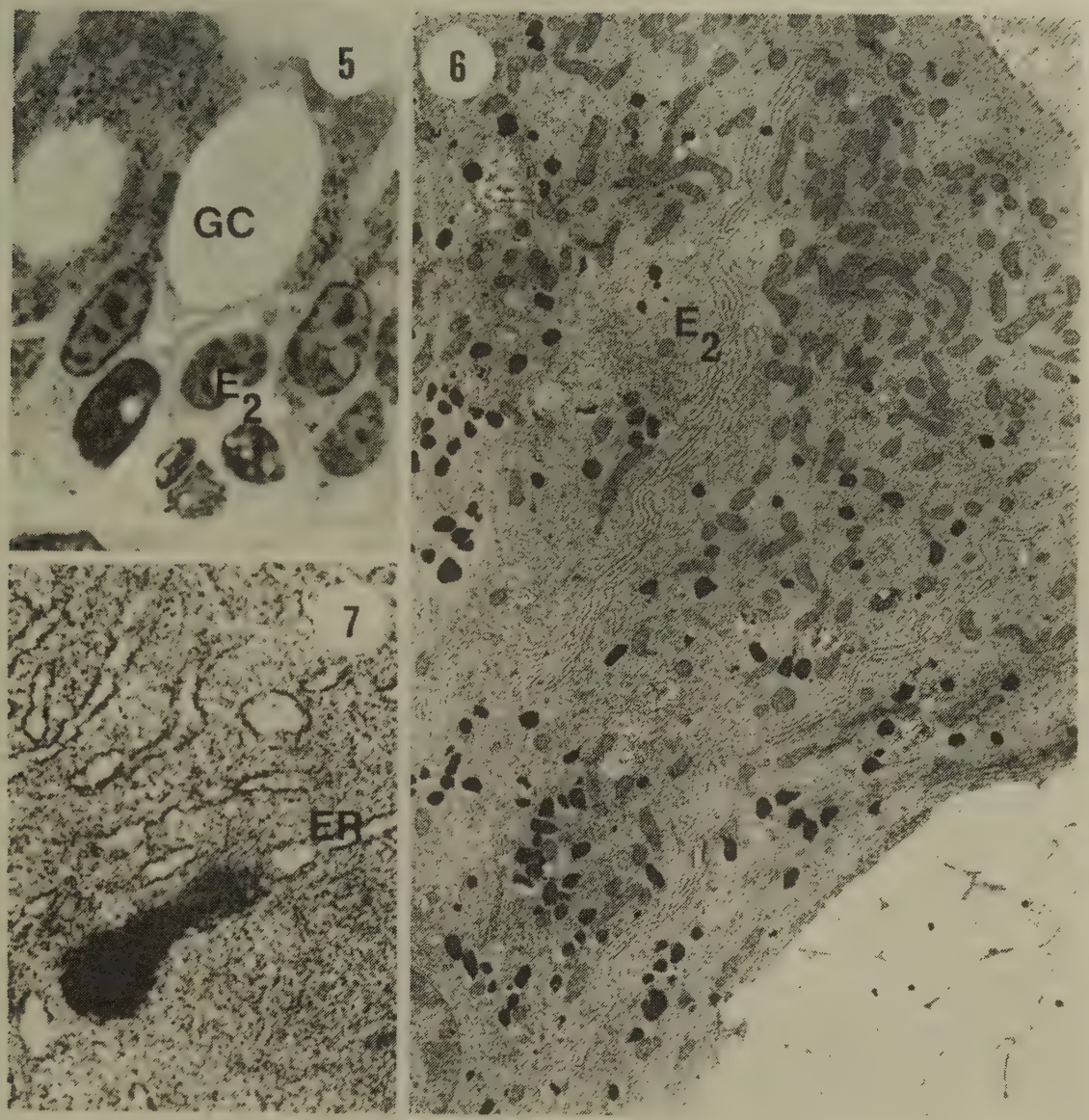


Fig. 5 Light micrograph of the intestinal epithelium ($E_2$) of an adult Rana catesbeiana. Goblet cells (GC) are fairly numerous. Most microperoxisomes are seen in the supranuclear area of the absorptive cells. X 350.

Fig. 6 In adult Rana catesbeiana microperoxisomes are numerous in the supranuclear area but they do not reach the terminal web; most of them are found in the lower two thirds of this zone. X 17,900.

Fig. 7 In adults most DAB-positive structures have a circular profile but occasionally a tubular DAB-positive structure may be observed. Here the membrane of the microperoxisome is in continuity with that of the smooth endoplasmic reticulum (ER). X 60,600.

DEVELOPMENT OF PEROXISOMES IN AMPHIBIANS.

II. CYTOCHEMICAL AND BIOCHEMICAL STUDIES ON THE LIVER, KIDNEY AND PANCREAS.

Journal of Experimental Zoology.

# DEVELOPMENT OF PEROXISOMES IN AMPHIBIANS

## II. Cytochemical and biochemical studies on the liver, kidney and pancreas.

M. DAUÇA [1,3], R. CALVERT [2], D. MENARD [2*], J.S. HUGON[2] and J. HOURDRY [3]

1. Laboratoire de Biologie et Biochimie du Développement
Ecole Normale Supérieure
Grille d'Honneur - Parc de Saint-Cloud
92210 - Saint-Cloud - France

2. Département d'anatomie et de biologie cellulaire
Unité de biologie intestinale
Faculté de Médecine - Université de Sherbrooke
Sherbrooke, Québec, Canada J IH 5N4

3. Laboratoire de Biologie Vertébrés
E.R.A. 902
Centre d'Orsay. Université Paris-Sud
91405 - Orsay - France

## ABSTRACT

The ontogeny of catalase-containing organelles was studied by cytochemical and biochemical methods in the liver, kidney and pancreas during the development of Rana catesbeiana. The biochemical differentiation of peroxisomes in the liver and kidney was compared to that of Xenopus laevis. Catalase activity was localized after incubation in DAB-medium and studied biochemically by a spectrophotometric method. In Rana catesbeiana the number of catalase-positive organelles per cell section is low in all three organs during premetamorphosis; their number increases substantially in the liver and kidney of froglets while it remains almost stable in the pancreas. No further increase is observed in the adult. Biochemically, the liver, kidney and pancreas of tadpoles exhibit respectively 12, 22 and 63% of the catalase activity found in the adult tissues. After metamorphosis an important increase of catalase activity is particularly noted in liver and kidney, the activity being respectively 43 and 77% of that adult bullfrogs. On the other hand, no change in catalase activity in the liver and kidney is noted during the entire development of Xenopus laevis. The present study illustrates the very different developmental pattern of catalase activity observed during the development of two anuran amphibians. The different developmental pattern of the same enzyme within the small intestine, liver, kidney and pancreas in Rana catesbeiana is also stressed.

# INTRODUCTION

Peroxisomes and microperoxisomes (characterized biochemically by the presence of $H_2O_2$ - generating oxidases and by an $H_2O_2$ - destroying catalase) have been described in a wide variety of animals principally in mammals (see review by Boëk et al. '80.) Peroxisomes or microperoxisomes have received little attention during the development of amphibians, especially during metamorphosis which is characterized by spectacular morphological alterations accompanied by histological and biochemical changes (Frieden and Just, '70; Dodd and Dodd, '76; Cohen et al, '78; Hourdry, '80.) Peroxisomes were described in the kidney and liver in the adult (Hruban and Recheigl, '69: Scott et al.,'69; Roels et al, '70); more recently, microperoxisomes were reported in the pancreas of adult Urodeles and Anura (Trandaburu, '80.) and in the epithelial cells of the urinary bladder of Bufo marinus (Jones et al, '81)

In the first paper of this series, we have reported the developmental pattern of microperoxisomes in the intestinal epithelial cells of two species of amphibians namely, Rana catesbeiana and Alytes obstetricans. After metamorphosis the absorptive cells of the new secondary intestinal epithelium showed a drastically increased number of microperoxisomes per cell section together with a concomitant rise in catalase activity (Dauça et al., '82.)

In the present paper, we report a systematic study on the developmental pattern of peroxisomes in the liver and kidney and of microperoxisomes in the pancreas of Rana catesbeiana together with a comparative study of catalasase activity in the liver and kidney of Xenopus laevis. It is to be remembered that before metamorphosis larvae of Rana catesbeiana are ammonotelic and that after metamorphosis when they adapt to their new terrestrial environment they become ureotelic. On the other hand, metamorphosis in Xenopus laevis tadpoles is not followed by a change in the mode of excretion and adults remain ammonotelic; furthermore no changement of habitat is observed in this species.

## MATERIALS AND METHODS

ANIMALS Rana catesbeiana and Xenopus laevis tadpoles (stages X - XII) and adults (2 years old) were purchased from McNally (Wisconsin, U.S.A.) Tadpoles were allowed to metamorphose at 20-25°C in the laboratory.

CYTOCHEMISTRY. After decapitation, the liver and kidney from Rana catesbeiana were removed and perfused with ice-cold 2.8% glutaraldehyde buffered with 0.1 M sodium cacodylate during 2 min. Then, small pieces of liver and kidney as well as pancreas were immersed for 30 min in the fixative solution and washed in 0.2 M sodium cacodylate buffer. Thick (40 µm) frozen sections were made and incubated at pH 9.7 during 3 hrs according to the method described by NoviKoff et al., ('72) as applied previously (Dauça et al., '82). For cytochemical controls, the inhibitor 3-amino-1,2,4-triazole (Sigma Chemical Co., St-Louis, MO) was added to the complete medium. In addition, some sections were incubated after heat denaturation of the enzyme. Incubated sections were postfixed in 2% osmium tetroxide, dehydrated and then embedded in Epon. Thick Epon sections were stained during 1 min at 70°C with toluidine blue (1%) and sodium borate (1%). Thin sections were stained with uranyl acetate only or double stained with uranyl acetate and lead citrate (Reynolds, '63) and were examined in a Philips 300 or in a Jeol JEM 100 B electron microscope.

MORPHOMETRY The number of microperoxisomes per cell section was recorded in 20 cells from 2 animals by direct observation with the electron microcospe. The diameters of the DAB-positive organelles were measured on electron micrographs in 10 cells per developmental stage on electron micrographs enlarged 40,000 to 80,000 times.

BIOCHEMICAL METHODS. A comparative study of catalase activity was undertaken in liver and kidney of Rana catesteiana and Xenopus laevis, the pancreas being used only in Rana catesbeiana. After decapitation, liver and kidney were removed and perfused with ice-cold M/15 phosphate buffer, pH 7.0, in order to remove red blood cells. Then the kidney, liver and pancreas were washed again in buffer, blotted dry and weighed. The different tissues were homogenized in 10 vol of ice-cold phosphate buffer with a glass-Teflon homogenizer at 4°C. After homogenization, aliquots were taken for protein assays according to Lowry et al., ('51). Then, Triton X-100 was added to the homogenates to a final concentration of 1% as previously reported (Calvert and Ménard, '78; Dauça et al., '82). The homogenate was centrifuged at 5000 rpm/min during 10 min and the catalase activity was measured in the supermatant by the spectrophotometric method of Lück ('65). The enzymatic activity was expressed as units of catalase per g protein.

# RESULTS

## MORPHOMETRY

The number of DAB-positive organelles per cell section is reported in Figure 1. Observations were made in thin sections of liver, kidney and pancreas. The standard deviation was not calculated because the surface of the cells was not considered. In the premetamorphosis larvae the mean number of DAB-positive organelles per cell is very low in the cells of the pancreas and it is moderate in those of the kidney and liver. In froglets the number of microperoxisomes in pancreatic cells remains low; in the kidney and liver cells the mean number of peroxisomes increases substantially and no further increase was observed in the adult tissues. During development, the diameter of the DAB-positive structures remains stable in the pancreas and liver: it ranges from 200 to 300 nm in the former and from 500 to 900 nm in the latter. However, in the kidney it seems that the peroxisomes are slightly larger in the adult: in tadpoles the diameters vary from 400 to 500 nm in thickness whereas in the adult they measured from 500 to 830 nm in thickness.

## CYTOCHEMICAL OBSERVATIONS

The development of Rana catesbeiana is characterized not only by an increase in the number of peroxisomes in the liver cells but also by a darkening of the reaction product at the different stages, with identical incubation conditions: in tadpoles the reaction is faint, in froglets it

is moderate and in adults it is very strong (Figs 2-6). At all stages the peroxisomes are slightly ovoid or circular; DAB-positive tubular peroxisomes were never seen in the liver. Glycogen particles were dissolved or extracted in the liver sections during the treatments in the sodium cacodylate buffer (Fig. 6), especially in the adults (Leskes et al., '71)

In the kidney, peroxisomes are localized preferentially in the apical part of the cells at each developmental stage (Fig. 7-9). Furthermore, they are found excusively in the proximal tubules. Most of the kidney peroxisomes are ovoid (Fig. 10) In this organ the intensity of the reaction product is comparable between that observed in late metamorphosis tadpole (Fig. 10) and that in the adult (Fig. 11). Small DAB-positive organelles measuring 200 nm in diameter were often observed in collecting tubules; they were probably microperoxisomes.

The exocrine pancreas of tadpoles and froglets is characterized by its paucity in DAB-positive organelles. The diameter of these structures is always smaller than that of peroxisomes of the liver and kidney; they resemble the intestinal microperoxisomes. Most microperoxisomes have circular or ovoid profiles; some of them are seen in continuity with DAB-positive tubules (Fig. 12). In the pancreas the intensity of the cytochemical reactions is always faint, even in the adult. The cells of the duct system also contain a few DAB-positive organelles (Fig. 13). They are larger than those seen in acinar cells. They measure up to 400 nm in diameter and they are probably peroxisomes. The reaction product observed on these peroxisomes remains faint in the adult. With the cytochemical control specimens, DAB-positive structures were not seen in the incubated tissues.

CATALASE ACTIVITY

Table 1 illustrates the developmental patterns of catalase activity in different tissues of Rana catesbeiana and Xenopus laevis. At all developmental stages, the liver exhibits the highest catalase activity. In Rana catesbeiana tadpoles, the liver, kidney and pancreas exhibit respectively 12, 22 and 63% of the catalase activity found in adult tissues. After metamorphosis an important increase of catalase activity is particularly noted in liver and kidney, the activity being respectively 43 and 77% of that in adult bullfrogs. On the other hand, no change in catalase activity in the liver and kidney is noted during the entire development of Xenopus laevis.

# DISCUSSION

At climax of metamorphosis in amphibians the liver, kidney and pancreas are profoundly affected and they undergo important developmental changes. During these processes, the morphology of the liver is not affected by the renewing of the hepatocytes even though an important rise in DNA synthesis is occuring (Atkinson et al, '72; Kistler and Weber, '75; Smith-Gill,'79: Smith-Gill et al,'79). The hepatic parenchymal cells also differentiate rapidly during this period both morphogically (Bennett and Glem, '70; Spiegel and Spiegel, '70) and biochemically (Inaba and Frieden, '67; Ledford and Frieden, '73). We have shown herein that another organelle, the peroxisomes is affected by metamorphosis of Rana catesbeiana. The number of peroxisomes increases in the froglet; catalase activity increases steadily after metamorphosis and peaks in the adult. However in Xenopus laevis, catalase activity remains constant throughout development.

The kidney of anuran amphibians is also completely reorganized during post-embryonic development. The functional pronephros in the young tadpole will degenerate during metamorphosis and will disappear at the climax (Fox, '70). The mesonephros will appear precociously (Cambar and Williaume, '67; Spannhof and Jonas, '68) and its parenchyma will differentiate very rapidly and loose its capacity of proliferation in the juvenile. These changes in Rana catesbeiana are accompanied by an

increase of peroxisomes in the froglets; catalase activity also increases substantially in the froglets and afterwards remains almost stable. This behaviour is quite different from that observed in the liver of the same species. Here again in Xenopus laevis, catalase activity does not change after metamorphosis, a slight increase is noted in the adult.

Pancreas is probably the most affected organ during metamorphosis of Rana catesbeiana: it undergoes an important reduction in volume and a 80% decrease in weight (Atkinson and Little, '72). During spontaneous (Beaumont, '76) or induced (Slikers and Kim, '71) metamorphosis, the exocrine pancreas degenerates partially. In the adult, when the endorine and exocrine pancreas are fully differentiated, a very weak increase in the number of microperoxisomes and in catalase activity is observed.

It is now apparent that the developmental pattern of peroxisomes and microperoxisomes is a good marker to follow the differentiation of the epithelial cells of the intestine, kidney and liver of Rana catesbeina. However, it is worth noticing that the important changes observed in the specific activity of catalase and in the number of DAB-positive organelles are more precocious in the liver and kidney than in the intestine. In the latter the increases were observed in juveniles adapted to their new terrestrial environment for a few weeks when they were observed in larvae at the end of metamorphosis (Stages XXIV -XXV) in the kidney and liver.

The uprise in the number of peroxisomes and in catalase activity during metamorphosis in the liver, kidney and intestine of anuran amphibians is also observed in other vertebrates. In birds, it is observed at the time of hatching in the liver and kidney (Stephens and Bils, '67; Essner, '67). In mammals it is also noticed at time of birth

in the liver (Krahling et al., '79: David, '80) in the kidney (Goeckermann and Vigil, '75, Miles and Holmes, '75) and in the small intestine (Pipan and Psenicnik, '75; Calvert and Ménard '78) At present time, it is not known which factor (s) regulate(s) the differentiation of peroxisomes nor the precise role of these organelles in the physiology of these organs.

During the transition from froglets to adults the number of peroxisomes per cell sections of liver and kidney does not increase; during the same period however the activity of catalase increases in both organs: similar observations were made in the liver and kidney of mammals (Tsukada et al, '68; Goeckermann and Vigil, '75). Masters and Holmes ('77) have postulated that peroxisomes present in these organs at birth are more or less immature, and that they will fully differentiate only in the adult.

We have reported herein the very different developmental pattern of catalase activity observed during the development of Rana catesbeiana and Xenopus laevis. Other important differences were also reported between these two species. When metamorphosis is induced by thyroxine, the specific activities of the enzymes of the respiratory chain are not stimulated in Xenopus laevis (Kistler and Weber, '75) while they are in Rana catesbeiana (Cohen et al., '78). Furthermore, the ultrastructure of mitochondria is deeply affected by induced metamorphosis in Rana catesbeiana (Bennet et al., '70; Cohen, '70) while it is not in Xenopus laevis (Kistler and Weber, '75). These differences in the behaviour of peroxisomes and mitochondria are possibly related to the change in the mode of excretion in Rana catesbeiana: the ammonotelic larvae become ureotelic adults. Xenopus laevis remains ammonotelic throughout life.

Do peroxisomes and microperoxisomes play a role in this adaptation to a terrestrial habitat? Why catalase activity is lower in ammonotelic larvae of Rana catesbeiana? Further studies will be needed to elucidate these questions.

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TABLE 1

Developmental pattern of catalase activity during the development of Rana catesbeiana and Xenopus laevis.

| Developmental stage | Species | Catalase activity[a] | |
|---|---|---|---|
| | | liver | kidney |
| Premetamorphosis larvae | Rana c. | 8,470 ± 1,230[b] ( 9)[c] | 1,720 ± 170 ( 9) |
| | Xenopus l. | 52,620 ± 8,680 ( 9) | 22,950 ± 3,400 ( 9) |
| Juveniles (1-2 weeks) | Rana c. | 29,570 ± 2,990 (12) | 5,990 ± 510 (12) |
| | Xenopus l. | 42,150 ± 3,830 (13) | 21,600 ± 970 (13) |
| Adults (2 years) | Rana c. | 69,220 ± 9,440 ( 8) | 7,780 ± 920 ( 7) |
| | Xenopus l. | 60,370 ± 13,720 ( 5) | 33,670 ± 4,300 ( 5) |

a Expressed as units of catalase per g protein

b Mean ± SEM

c Number of assays

## FIGURE LEGENDS

Fig. 1 This figure illustrates the mean number of catalase-positive organelles per cell section in the pancreas, kidney and liver during development of Rana catesbeiana. (T: tadpoles, stages X - XII; F: froglets, stages XXIV - XXV; adults, 2 years old).

Fig. 2 Light micrograph of the liver parenchyma of a tadpole of Rana catesbeiana (stages X - XII). At this stage, DAB-positive organelles are very difficult to identify with the light microscope. X 100.

Fig. 3 At the same stage (X - XII), at the fine structural level, a few DAB-positive peroxisomes are present in hepatic parenchymal cells (arrow). The reaction is faint. X 24,000.

Fig. 4 At the end of metamorphosis (stages XXIV - XXV) ovoid peroxisomes are more numerous in the hepatic parenchymal cells of Rana catesteiana (arrow). X 19,500.

Fig. 5 In the adult liver, peroxisomes are easily seen even in thick Epon sections. X 250.

Fig. 6 Also in the adult liver of Rana catesbeiana, the reaction product on peroxisomes is very dark and these organelles are more numerous than in earlier stages. Glycogen was extracted during the cytochemical procedure. X 14,000.

Figs. 7-9 Thick Epon section showing the development of the mesonephros in a premetamorphosis larva of Rana catesbeiana (Fig. 7, X 160), in a larva at the end of metamorphosis (Fig. 8, X 140) and in the adult (Fig. 9, X 80) DAB-positive peroxisomes are present in the proximal tubules only (arrows).

Fig. 10 At the end of metamorphosis, peroxisomes are found mainly in the apical part of the cells (arrow) in the proximal tubules of the kidney, near the striated border (b) X 11,500.

Fig. 11 In the adult kidney peroxisomes are numerous and they show circular to elongated profiles (arrow). X 22,500.

Fig. 12 In the premetamorphosis larvae, microperoxisomes are not frequently observed in the cells of the exocrine pancreas of Rana catesbeiana. A few microperoxisomes are seen in continuity with DAB-positive tubular structures (arrow) X 46,000.

Fig. 13 In the adult, cells of the duct system in the pancreas also have peroxisomes (arrow) located close to the apical cell membrane. The intensity of the cytochemical reaction is low. X 23,000.

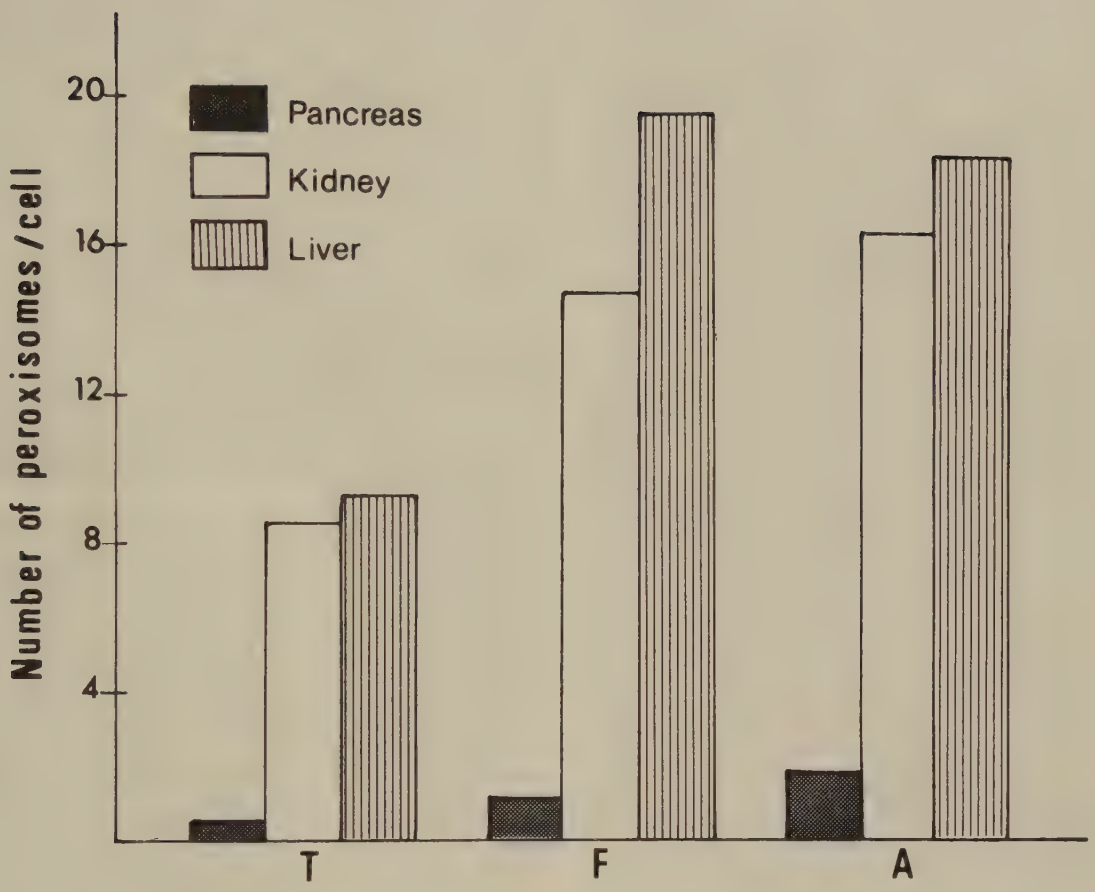
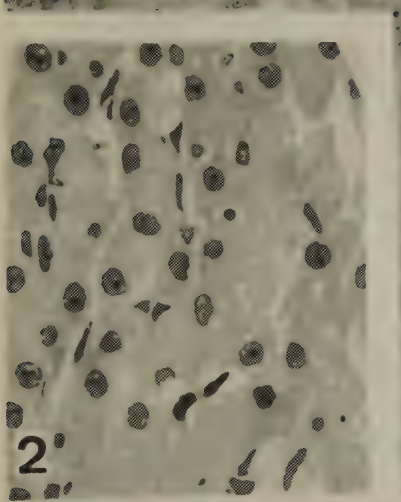



**Fig.1. The mean number of peroxisomes per cell.**

(T: tadpole, stages x. xii., F: froglet, stages xxiv. xxv., A: adult, 2 years-old).



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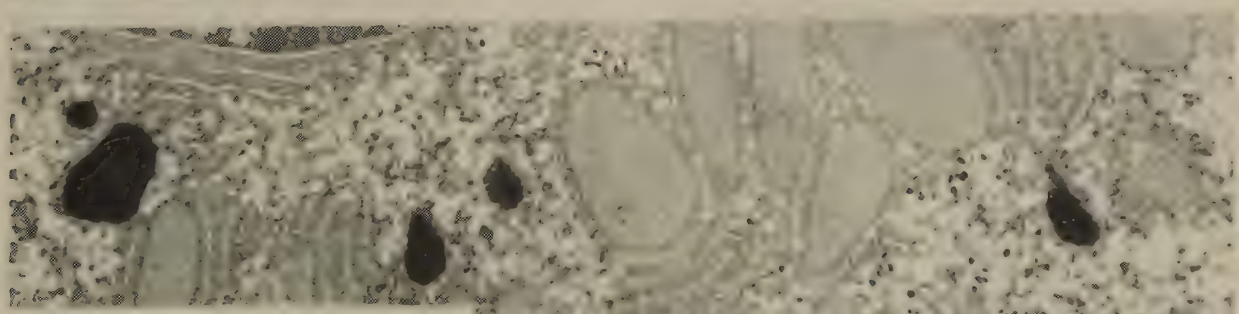

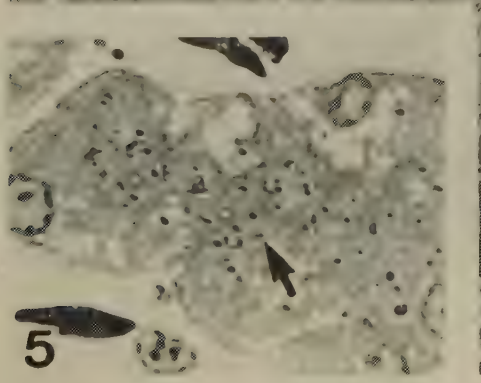

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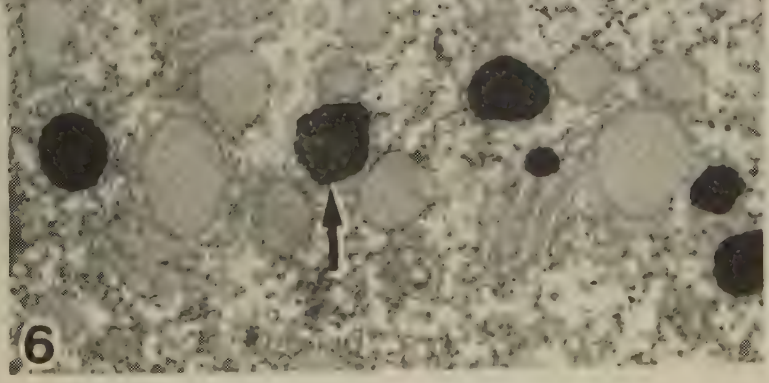

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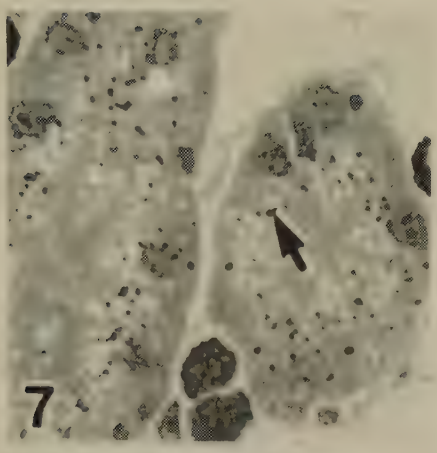
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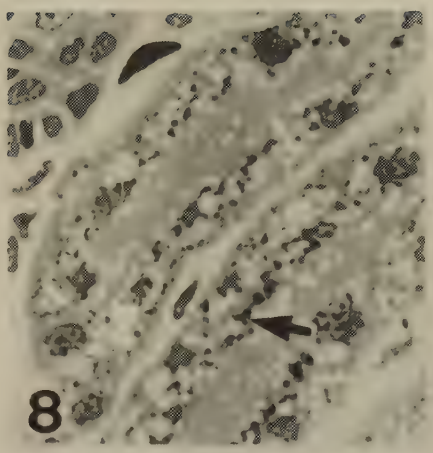
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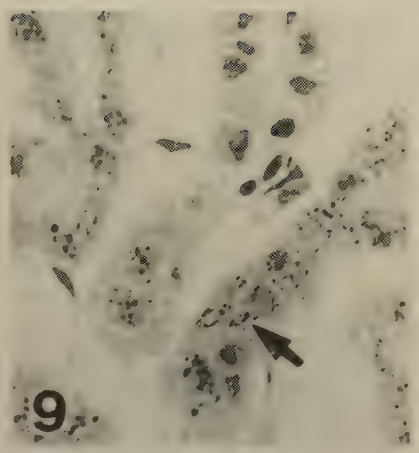
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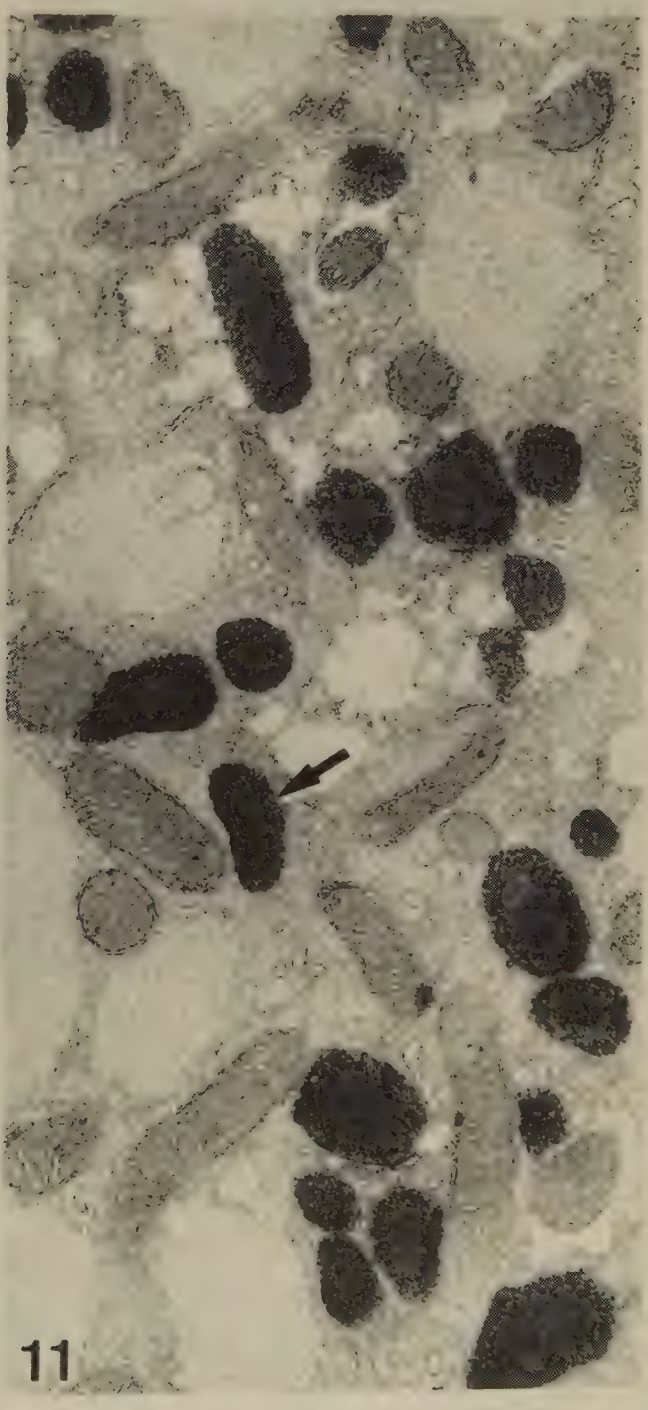
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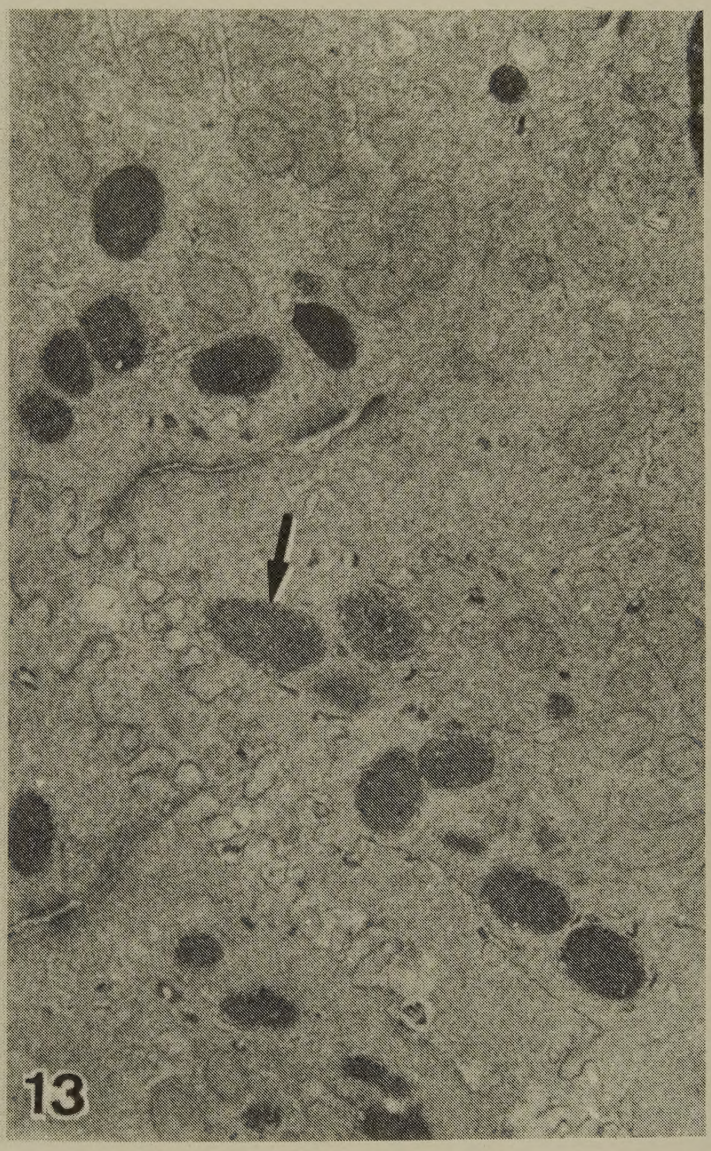
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